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THE BIOSYNTHESIS OF CELL WALL CARBOHYDRATES

IV. FURTHER STUDIES ON CELLULOSE AND XYLAN IN WHEAT[1]

A. C. Neish

Abstract

D-Glucose-1-C^{14}, D-glucose-6-C^{14}, D-mannose-1-C^{14}, D-galactose-1-C^{14}, D-glucuronolactone-1-C^{14}, D-glucuronolactone-6-C^{14}, potassium D-gluconate-6-C^{14}, and L-arabinose-1-C^{14} were administered to wheat shoots. The cellulose and xylan were isolated after a 5 hour period of metabolism. Glucose was more readily converted to cellulose and xylan than any of the other compounds tested. The distribution of C^{14} in the glucose and xylose isolated from the polysaccharides indicates that xylan was formed from the aldohexoses and glucuronolactone by processes involving loss of carbon-6. L-Arabinose, unlike D-xylose and D-ribose, was converted to xylan with little rearrangement of the pentose skeleton.

Introduction

In previous papers of this series (1, 2, 4, 18) it has been shown that glucose administered to shoots of the wheat plant is incorporated into cellulose and xylan more readily than any of the other carbohydrates tested. The conversion of glucose to cellulose took place with little rearrangement (about 20%) of the carbon skeleton. The xylan was apparently derived by processes involving loss of carbon-6 of the hexose. Similar results have been reported for wheat seedlings by Edelman, Ginsburg, and Hassid (5) and by Ginsburg and Hassid (8). In an investigation of pectin formation from C^{14}-labelled hexoses in the boysenberry (20) and strawberry (21), Seegmiller and coworkers found that L-arabinose was apparently formed by loss of carbon-6. This has also been shown to occur in wheat seedlings (8).

D-Ribose and D-xylose are not as readily converted to xylan as is glucose (1, 2, 18). Xylan is formed from these pentoses with considerable rearrangement of the pentose carbon skeleton, such as would occur if the pentoses were first converted to hexose by the action of transketolase and transaldolase (1, 2). Uronic acid derivatives may function as intermediates in the conversion of hexose to pentose precursors of xylan (1, 2) or pectin (20, 21).

In this paper further experiments are reported which extend and confirm the conclusions of earlier work. In addition it has been found that L-arabinose, unlike D-xylose or D-ribose, can be converted to xylan with little rearrangement of the carbon skeleton.

[1]Manuscript received September 26, 1957.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.
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Experimental

Materials

D-Glucose-1-C^{14} and D-manose-1-C^{14} were purchased from Atomic Energy of Canada. D-Glucose-6-C^{14}, D-galactose-1-C^{14}, and D-glucuronolactone-6-C^{14} were obtained from Merck and Co., Montreal. Potassium D-gluconate-6-C^{14} was prepared by hypoiodite oxidation (15) of D-glucose-6-C^{14}, by Dr. E. R. Blakley. L-Arabinose-1-C^{14} was prepared by the cyanohydrin synthesis from $KC^{14}N$ (7, 17) using a sample of L-erythrose furnished by Dr. P. A. J. Gorin and made by lead tetraacetate oxidation of L-glucose (19).

Growth and Activation of Plants

Thatcher wheat was grown in subirrigated gravel culture (6) using a modified Hoagland's solution (13). The solution was stored in a reservoir made from polyethylene, and pumped up (for 5 minutes) every 6 hours.

The plants were illuminated for 16 hours daily at an intensity of 1500 ft-c. Tungsten lamps provided one-third of the light intensity and cool white fluorescent tubes the remainder. The night temperature was 65° F. and the day temperature about 75° F. Healthy plants of uniform size were obtained.

The largest tillers from plants 41 days old (calculated from date of seeding) had an average dry matter content of 0.31 g. while those from 50-day-old plants contained 0.57 g. The heads were just starting to emerge in the 50-day-old plants. Eight tillers were selected for each experiment (see Table I). They were removed from the plant by cutting under water, and the labelled compounds administered as described previously (18). A period of 5 hours was allowed for metabolism (at 22° C. and 400 ft-c.) of the labelled compounds in all experiments.

Isolation and Degradation of the Products

When the 5 hour metabolic period was finished the fresh plant material was cut into short lengths (about 2 cm.) with scissors and blended with 200 ml. of hot 80% ethanol in a Vir Tis homogenizer. The fibrous residue was collected on a Buchner funnel and washed with hot 80% ethanol, then with hot absolute ethanol, and finally with ether. The almost colorless residue was then converted to holocellulose and fractionated into cellulose and xylan as in previous work (18). These polysaccharides were hydrolyzed, the sugars degraded by fermentation with *Leuconostoc mesenteroides*, and the C^{14} content of the products determined as before (1).

The free sugars (glucose, fructose, and sucrose) were isolated from the ethanolic extract in one experiment (No. 1, Table I), and their specific activities determined (see Table II). The ethanolic extract was evaporated to dryness by an air stream at room temperature, the residue was extracted with hot water, filtered through celite, and the filtrate deionized by Amberlite IR-120 and IR-4B resins. The deionized solution was divided into monosaccharide and disaccharide fractions by a charcoal column (24). The monosaccharides were further fractionated on a cellulose column with phenol-water (3) to give glucose and fructose fractions. The disaccharide fraction

appeared to contain sucrose as the only sugar when chromatographed on paper with *n*-butanol – acetic acid – water (4:1:1.8), using *p*-anisidine hydrochloride spray reagent. The glucose and fructose fractions examined in the same way also appeared to be pure. They were dissolved in water and a small portion of each fraction was analyzed by the anthrone method (16). Carrier was then added (10 parts for the monosaccharides and four parts for sucrose) and the sugars crystallized from ethanol–water mixtures. The specific activities in Table II are calculated on a carrier-free basis.

Results

The relative effectiveness of the labelled compounds as precursors of cellulose and xylan is shown by the last two columns in Table I. Glucose was considerably more effective than any other compound. Mannose and gluconate were poor precursors of both cellulose and xylan but L-arabinose and D-glucuronolactone-1-C^{14} were fairly good precursors of xylan though poor precursors of cellulose. In the younger plants xylan synthesis appeared to be proceeding at a more rapid rate, relative to cellulose formation, than in the older plants.

The water-soluble constituents of the ethanol extract accounted for 51.5% of the C^{14} administered in experiment 1, while the holocellulose contained 22.4%. The three sugars (glucose, fructose, and sucrose) accounted for only 65% of the C^{14} in the water soluble constituents (Table II). Another large fraction may have been present as fructosans (14), but this possibility was not investigated. Most of the C^{14} (91%) in the sugars was found in the sucrose. Although glucose-1-C^{14} was fed, the sucrose isolated had 5.1 times the specific activity of the free glucose in the same plant at the end of the experiment.

The distribution of C^{14} in the free glucose and the cellulose–glucose of the same plant (Table III) shows that the same rearrangement from carbon-1 to carbon-6 has occurred as shown in previous reports (1, 2, 4, 5, 18, 22). However the free glucose is not rearranged quite as much and still retains 88% of the C^{14} in carbon-1. D-Mannose-1-C^{14} shows about the same amount of rearrangement as glucose and D-galactose a little more. The figures for galactose are close to those obtained for its conversion to the glucose moiety of sucrose in wheat seedlings (12). The experiment with D-glucose-6-C^{14} shows that the rearrangement also occurs from carbon-6 to carbon-1 to about the same extent. This has also been found for cotton bolls (22) and wheat seedlings (5).

The 1-C^{14} aldohexoses and glucuronolactone all gave rise to xylan through processes which appear to involve loss of carbon-6. This confirms previous work with glucose (1, 2, 8, 18) and glucuronolactone (1, 2). L-Arabinose-1-C^{14} was converted to xylan with little rearrangement of the pentose carbon skeleton, 87% of the C^{14} being still retained in carbon-1. In this respect it differs markedly from D-xylose and D-ribose, which are extensively rearranged during conversion to xylan (1, 2, 18).

TABLE I

INCORPORATION OF C^{14} INTO CELLULOSE AND XYLAN BY WHEAT SHOOTS METABOLIZING LABELLED CARBOHYDRATES

Expt. No.	Compound fed	Dose per g. of dry plant material: μM. of compound	Dose per g. of dry plant material: mμc. of C^{14}	Age of plant* (days)	% of C^{14} fed in holocellulose	mμc./mM. of carbon: Cellulose	mμc./mM. of carbon: Xylan	Relative specific activity† (×10,000): Cellulose	Relative specific activity† (×10,000): Xylan
1	D-Glucose-1-C^{14}	29.3	8340	50	22.4	115	118	24.0	24.6
2	D-Glucose-6-C^{14}	26.6	1770	50	18.3	22.8	11.0	20.8	10.0
3	D-Mannose-1-C^{14}	28.2	2730	50	2.56	4.71	6.17	2.92	3.82
4	D-Glucuronolactone-6-C^{14}	35.1	4400	50	0.63	1.00	2.91	0.48	1.39
5	Potassium D-gluconate-6-C^{14}	30.3	2060	50	5.33	4.02	3.75	3.55	3.32
6	D-Glucose-1-C^{14}	50.6	14,500	41	16.7	175	253	36.6	53.0
7	D-Galactose-1-C^{14}	46.0	5700	41	3.72	11.4	26.7	5.33	12.9
8	L-Arabinose-1-C^{14}	59.5	1040	41	3.80	1.33	4.98	3.80	14.3
9	D-Glucuronolactone-1-C^{14}	51.0	955	41	4.38	1.01	6.11	3.24	19.6

*Age was calculated from date of seeding.

†Specific activity of carbon dioxide from complete combustion of polysaccharide divided by the specific activity of the carbon dioxide obtained by combustion of the original labelled carbohydrate.

TABLE II

CONVERSION OF GLUCOSE-1-C^{14} INTO FREE SUGARS (EXPT. 1, TABLE I)

Sugar isolated	mg./g. dry plant material	mμc./mM. of carbon	Total mμc. of C^{14} recovered
Glucose	5.4	737	648
Fructose	5.9	577	501
Sucrose	18.9	3850	12,420
Total water-soluble constituents	—	—	21,200

TABLE III

DISTRIBUTION OF C^{14} IN GLUCOSE AND XYLOSE ISOLATED FROM THE CELL WALL POLYSACCHARIDES

Expt. No. *	Compound fed	Sugar isolated	C^{14} as % of total in glucose as xylose molecule					
			Carbon -1	Carbon -2	Carbon -3	Carbon -4	Carbon -5	Carbon -6
1	D-Glucose-1-C^{14}	Glucose†	88.0	1.4	1.2	1.0	0.7	7.7
		Glucose	80.0	1.5	2.3	2.3	0.3	13.6
		Xylose	93.6	0.9	3.0	1.4	1.1	—
6	D-Glucose-1-C^{14}	Glucose	79.5	2.3	2.2	1.3	0.3	14.4
		Xylose	94.0	0.8	2.7	1.3	1.2	—
3	D-Mannose-1-C^{14}	Glucose	82.5	3.7	3.2	1.8	0.5	8.3
		Xylose	86.4	2.8	2.2	3.5	5.1	—
7	D-Galactose-1-C^{14}	Glucose	73.4	5.9	2.3	1.4	1.4	15.6
		Xylose	91.0	2.3	2.5	1.7	2.5	—
2	D-Glucose-6-C^{14}	Glucose	10.6	1.7	1.4	1.2	0.4	84.7
		Xylose	63.0	3.7	12.0	5.0	16.3	—
9	D-Glucurono-lactone-1-C^{14}	Glucose	17.7	26.2	14.7	2.7	21.0	17.7
		Xylose	89.0	3.0	1.9	2.2	3.6	—
4	D-Glucurono-lactone-6-C^{14}	Glucose	36.4	17.8	14.0	15.6	6.7	9.4
		Xylose	30.7	19.7	11.5	23.2	14.9	—
5	Potassium D-gluconate-6-C^{14}	Glucose	21.4	5.3	3.3	1.6	2.2	66.2
		Xylose	64.1	6.9	5.6	7.6	15.8	—
8	L-Arabinose-1-C^{14}	Glucose	30.2	18.8	11.0	17.6	9.6	12.8
		Xylose	87.0	1.6	1.4	3.4	6.6	—

*Experiment number the same as in Table I.
†This sample is the free glucose isolated from the water soluble fraction (see Table II).

D-Glucuronolactone-6-C^{14} can give rise to cellulose by indirect minor pathways. D-Gluconate-6-C^{14} can also form cellulose and here about 66% of the C^{14} is retained in carbon-6. This suggests reversal of reactions of the hexosemonophosphate shunt. The xylan formed from gluconate may have been formed from the hexose phosphate pool since loss of carbon-6 seems to be indicated.

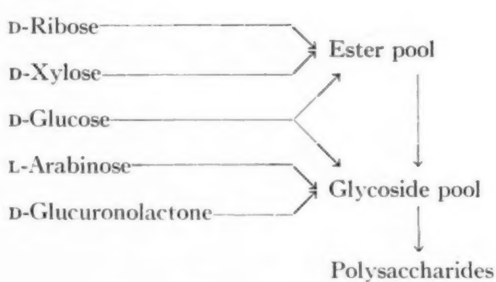
Discussion

The results of this investigation support conclusions of previous work. The carbon-1 to carbon-6 rearrangement of the hexoses is quite evident and has been shown to occur in both directions as has been found in cotton bolls (22) and in wheat seedlings (5, 12). This supports the conclusion that Embden–Meyerhof glycolytic reactions are responsible (5).

The formation of xylan precursors from hexoses and glucuronolactone by loss of carbon-6 has again been demonstrated. However the xylan formed from glucose-6-C^{14} is more strongly labelled than might have been expected, although the preponderance of C^{14} in carbon-1 of the xylose isolated can be predicted on the assumption that the carbon-1 to carbon-6 rearrangement has occurred to a noticeable extent before loss of carbon-6. The presence of quite an appreciable amount of C^{14} in carbon-5 of the xylose formed from glucose-6-C^{14} suggests that some of the pentose for xylan synthesis has been derived from the hexosemonophosphate shunt.

The preferential incorporation of glucose (fed through the stems) into sucrose rather than into the monosaccharide pool agrees with the experiments of Hassid and co-workers, who fed glucose (5) and galactose (12) to wheat seedlings through the roots. This result has been explained by assuming phosphorylation to be linked with absorption into living cells (5).

The incorporation of L-arabinose into xylan with little rearrangement of the carbon skeleton is interesting since D-xylose itself was converted to xylan with considerable rearrangement (1, 2, 18). A possible explanation for this may be suggested by considering the following hypothetical outline of polysaccharide synthesis.



Polysaccharides would be expected to be formed by transglycosylation reactions from some glycoside such as corresponding hexose- or pentose-1-phosphates. It is possible that nucleotides are involved and in previous papers of this series (1, 2) evidence for the participation of the uridine diphosphate derivatives has been summarized. Further support for this concept is given by the presence of the uridine diphosphate glycosides of D-xylose, L-arabinose, and D-glucuronic acid in mung bean seedlings (10, 23) and by the demonstration of a uridyl transferase and a waldenase which together can convert D-xylose-1-phosphate to a mixture of the uridine diphosphate glycosides of D-xylose and L-arabinose (9). Furthermore it has been shown recently (11) that uridine diphosphate glucose is used as a glucosyl donor for cellulose synthesis by a cell-free extract of *Acetobacter xylinum*.

The glycoside pool in the above diagram then may be considered as composed of glucose-1-phosphate, uridine diphosphate glycosides, sucrose, and possibly pentose-1-phosphates. The ester pool is intended to represent the

pentose-5-phosphates, glucose-6-phosphate, sedoheptulose-7-phosphate, and other compounds involved in the hexosemonophosphate shunt (i.e. substrates for aldolase, transaldolase, and transketolase).

When a labelled sugar is fed to a living plant the actual result will depend to a large extent on which of these pools it enters most readily. Glucose is apparently absorbed as hexose phosphate and due to the rapid interconversion of the 1- and 6-phosphate esters (by phosphoglucomutase) it can enter both pools readily. The actual mechanism of absorption and activation of the pentoses is not well understood but apparently D-ribose and D-xylose enter the ester pool and are metabolized there most readily; they appear to enter the glycoside pool with difficulty, the easiest route seems to be through hexose phosphate (1, 2, 18). L-Arabinose on the other hand seems to enter the glycoside pool most readily. Here it may be converted through unknown intermediates to the uridine diphosphate arabinose which, under the influence of a waldenase, is converted to uridine diphosphate xylose (9). The latter has been postulated as a precursor of xylan (1). This interpretation is necessarily highly speculative owing to lack of knowledge concerning the activation of pentoses and the biological significance of pentose-1-phosphates.

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A MODIFIED PROCEDURE FOR THE ESTIMATION OF VITAMIN B_{12} IN NATURAL MATERIALS OF LOW POTENCY[1]

J. M. McLaughlan, C. G. Rogers, E. J. Middleton, and J. A. Campbell

Abstract

The isotope dilution assay for vitamin B_{12} depends on the extraction and removal of vitamin B_{12}-like factors prior to the spectrophotometric measurement of vitamin B_{12}. Since it is not suitable for low potency materials, modifications have been made in the method of extraction and purification. Vitamin B_{12} is measured with *Lactobacillus leichmannii* 313. The purification procedure was effective in removing pseudovitamin B_{12} and factor A, but was ineffective in the removal of vitamin B_{12} III. A correction factor was used in place of Co^{60}-labelled vitamin B_{12} to adjust for loss during purification. Statistical analysis showed this correction factor to be valid and reproducible. Purification did not affect the estimates of vitamin B_{12} activity of milk, blood plasma, chick mash, or fish meal. True vitamin B_{12} activity was apparently responsible for 50% of the total vitamin B_{12} activity of dried cattle faeces and for less than 10% of the total activity of yeast extract. The coefficient of variation of the modified method was 8.7% based on 20 potency estimates.

Introduction

Existing methods for the estimation of vitamin B_{12} in low potency materials frequently lack specificity and are often subject to considerable variation. The *Lactobacillus leichmannii* assay for vitamin B_{12} has been adopted as the official method by the U.S.P. (10) and the A.O.A.C. (8). It does not, however, give reliable results in the presence of desoxyribosides and certain of the vitamin B_{12}-like factors to which *L. leichmannii* responds. Recent reports (3,7,12) have shown the rather widespread occurrence of vitamin B_{12} analogues in natural materials, particularly in those which have been subjected to microbial fermentation. These reports have emphasized the need for assay methods that are specific for vitamin B_{12}. The *Ochromonas malhamensis* assay (5) is probably the most specific of the microbiological procedures, but has certain disadvantages. A 72 hour incubation period is required, during which the cultures must be shaken continuously; also many workers are unfamiliar with the techniques required for protozoan assays. It has recently been reported (4) that the *Ochromonas* assay is not reliable in the presence of vitamin B_{12} III.

Bacher, Boley, and Shonk (1) proposed a radioactive tracer assay for vitamin B_{12} which has been adopted by the U.S.P. (2, 11). The method is considered to be specific for vitamin B_{12} but has not been found applicable to materials of low potency because of the necessity of measuring the vitamin B_{12} spectrophotometrically. The present paper describes a modification of the method of Bacher *et al.* that is suitable for the measurement of vitamin B_{12} activity in low potency materials.

[1]Manuscript received September 12, 1957.
Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada. A preliminary account of this work was presented at the 21st Annual Meeting of the American Institute of Nutrition, Chicago, Ill., U.S.A., April 15–19, 1957.

Experimental

Reagents

(1) Sodium metabisulphite – phosphate solution (11).
(2) Cresol – carbon tetrachloride solution (11).
(3) Sulphuric acid, 5 *N*.
(4) Phosphate–cyanide solution (11).
(5) Butanol – benzalkonium chloride solution (11).

Extraction of Samples

Autoclave approximately 1 g. or 1–3 ml. of sample for 15 minutes (121–123° C.) with 20 ml. of the bisulphite–phosphate extracting solution in a 40 ml. Maizel-Gerson reaction vessel. Cool, and centrifuge for 1 minute. Quantitatively transfer the supernatant liquid to a second 40 ml. Maizel-Gerson flask, washing the residue with 5 ml. of distilled water.

Purification Procedure

The method of purification is similar to that described in the U.S.P. (11) although several steps have been omitted. The procedure is carried out as follows:

Add 5 ml. of the cresol – carbon tetrachloride solution to the bisulphite–phosphate extract and shake the mixture gently for 1 minute. *Avoid* strong agitation to prevent possible gel formation. Centrifuge for 2 minutes at not less than 3000 r.p.m. With a hypodermic syringe, remove and discard the upper aqueous phase taking care not to remove any of the solid layer which may form at the interface of the two solvents. Wash the organic solvent phase with 5 ml. of 5*N* sulphuric acid. Shake the flask gently for 1 minute and centrifuge for 2 minutes; remove and discard the upper aqueous phase. Repeat once the wash with sulphuric acid. Add 5 ml. of the phosphate–cyanide solution to the organic solvent phase. Shake the mixture gently for 1 minute, centrifuge for 2 minutes, and remove and discard the upper aqueous phase. To the washed organic phase add 15 ml. of 2 : 1 butanol – benzalkonium chloride : carbon tetrachloride solution and 5 ml. of distilled water. Shake the mixture gently for 1 minute, centrifuge for 2 minutes, and remove and *retain* the upper aqueous phase. Wash the residual organic layer a second time with 5 ml. of distilled water and remove and combine the upper aqueous layer with the previous aqueous extract.

Dilute the combined aqueous extract with distilled water to give a final solution containing a concentration of vitamin B_{12} activity equivalent to approximately 0.02 millimicrogram of cyanocobalamin per milliliter. Measure the activity of this solution by microbiological assay with *L. leichmannii* 313.

For samples known to be of very low vitamin B_{12} activity wash the combined aqueous extract with 5 ml. of ethyl ether to remove traces of organic solvents which can interfere with growth of the test organism. With materials of greater vitamin B_{12} activity, this interference is eliminated by dilution. Mix the solutions *very gently* during ether extraction to avoid gel formation.

In experiments to determine the expected percentage loss of vitamin B_{12} during purification, cyanocobalamin labelled with Co^{60}* was incorporated with the samples and carried through the procedure. The amount of radioactivity present before and after purification was measured with a scintillation counter equipped with a scintillometer tube set up for liquid counting.

Results

The Effect of Purification on the Removal of Vitamin B_{12}-like Factors

Preliminary tests were made with the modified method to determine the efficiency of removal of vitamin B_{12}-like factors by purification. The results of these tests are summarized in Table I. Purification removed pseudovitamin B_{12} in the presence of vitamin B_{12} as shown by the results of experiment 1. In experiment 2, before purification, factor A was approximately 20% as active as vitamin B_{12} in promoting growth, but factor B was inactive. Pseudovitamin B_{12} and vitamin B_{12} III were each about 50% as active as vitamin B_{12}. The activity of each factor remaining after purification was compared against an untreated standard of the same factor. Purification removed approximately 75% of factor A and 90% of pseudovitamin B_{12}, but only about 25% of vitamin B_{12} III.

TABLE I

EFFECT OF PURIFICATION ON THE ACTIVITY OF VITAMIN B_{12}-LIKE FACTORS

Compound	Approximate activity for *L. leichmannii*[a] Before purification	After purification
Experiment 1		
Cyanocobalamin	100	91
Cyanocobalamin + pseudovitamin B_{12}[b]	144	92
Experiment 2		
Factor A	20	5
Factor B	0	0
Pseudovitamin B_{12}	50	5–10
Vitamin B_{12} III	50	35

[a]Expressed as % of activity of an equivalent amount of vitamin B_{12}.
[b]Ratio of pseudovitamin B_{12} to cyanocobalamin was 1.5:1.
NOTE: The pseudovitamin B_{12} used in Experiment 1 was obtained from Merck and Co., Rahway, N.J., U.S.A., and that used in Experiment 2 was obtained from Dr. M. E. Coates, National Institute for Research in Dairying, Reading, England.

Derivation of a Correction Factor

A correction factor was established to account for loss of vitamin B_{12} during purification. This was done to replace the use of Co^{60}-labelled vitamin B_{12} tracer solution. A measured quantity of the Co^{60} tracer was added to each of nine widely different natural materials. Samples were extracted with

*A solution of cyanocobalamin, made radioactive by the incorporation of Co^{60}, was obtained from Merck and Co. Inc., Rahway, N.J., U.S.A.

TABLE II

PERCENTAGE RECOVERY OF ADDED Co^{60}-LABELLED VITAMIN B_{12} AFTER PURIFICATION OF NATURAL MATERIALS

Material	No. of determinations	Mean recovery, %	Coefficient of variation
Blood plasma (steer)	12	94.3	$\pm$ 3.25
Fish meal	21	94.2	$\pm$ 4.91
Chick mash	21	94.1	$\pm$ 4.72
Sprouted peas (dried)	12	92.1	$\pm$ 5.39
Cattle faeces (dried)	21	94.7	$\pm$ 5.76
Urine (human)	17	93.7	$\pm$ 3.59
Bacterial cells (*E. coli*)	7	92.2	$\pm$ 6.63
Yeast extract (Difco)	3	94.7	$\pm$ 1.68
Milk	32	88.8	$\pm$ 7.02
Total (less milk)	114	93.8	Correction factor = $\frac{100}{93.8} = 1.07$

metabisulphite solution and the radioactivity of the extracts was measured before and after purification. In this way an estimate of the expected percentage recovery of vitamin B_{12} was obtained from the amount of radioactive tracer recovered. All percentage recovery values were corrected for purity of the cobalamin tracer solution (11). The results of the recovery tests with added Co^{60}-labelled vitamin B_{12} are summarized in Table II.

The mean recovery values for all samples were in close agreement with the exception of that for milk, which was consistently lower by about 5%. With values for milk omitted, the mean recovery value for all other samples was 93.8%, based on 114 determinations. This value was used to calculate a correction factor of 1.07 which was used to correct for loss of vitamin B_{12} during purification.

Validity of the Correction Factor

Experiments were carried out to test the validity of the use of a correction factor when applied to the modified procedure. Radioactive vitamin B_{12} was added to several natural materials from which the tracer was recovered by extraction with metabisulphite solution. The radioactivity of the extracts was measured before and after purification. Samples were treated in random order by three workers on each of 3 or 4 days and the data so obtained were subjected to statistical analysis. Analyses of variance of data from the tracer recovery experiments are summarized in Table III. In experiment 1, the tracer was recovered from extracts of blood plasma, fish meal, sprouted peas, chick mash, and dried cattle faeces by each of three workers on 4 days. In experiment 2, the tracer was recovered from extracts of fish meal, chick mash, dried cattle faeces, and urine by three workers on each of 3 days. In neither the first nor the second experiment was there a significant variation due to days or to any of the interactions. In experiment 1, a significant difference between workers was encountered at the 1% level of probability. In the

TABLE III

ANALYSES OF VARIANCE OF DATA FROM TRACER RECOVERY EXPERIMENTS

Variation due to:	Experiment 1 D.F.	Experiment 1 Mean square	Experiment 2 D.F.	Experiment 2 Mean square
Samples	4	10.78	3	34.04*
Workers	2	123.32**	2	24.11
Days	3	32.99	2	35.23
Samples × workers	8	13.34	6	14.05
Samples × days	12	23.62	6	20.24
Workers × days	6	13.76	4	24.86
Samples × workers × days (error)	24	12.53	12	9.36
Total	59		35	

*Significant at $P = .05$.
**Significant at $P = .01$.

second experiment the difference between workers was not statistically significant. A higher centrifuging speed (3000 r.p.m. rather than 2000 r.p.m.) was used in the second experiment to partition solvent layers, and this appeared to minimize the variation due to workers. In experiment 2, the variation due to samples was just significant at the 5% level of probability.

The Effect of Purification on Vitamin B_{12} Activity

A study was made to determine the effect of purification on the apparent vitamin B_{12} activity of several different natural materials. The samples were prepared by extraction with metabisulphite solution and the vitamin B_{12} activity of the extracts was determined microbiologically before and after purification. The proposed correction factor was applied to all potency estimates obtained after purification to correct for loss of vitamin B_{12}. The results of this study are summarized in Table IV. Purification did not significantly affect estimates of the vitamin B_{12} activity of extracts of milk, blood

TABLE IV

EFFECT OF PURIFICATION ON THE APPARENT VITAMIN B_{12} POTENCY OF NATURAL MATERIALS

	Millimicrograms per g. or per ml.						
	Before purification		After purification[a]				
Sample assay	1	2	1	2	3	4	5
Milk	2.8	2.6	2.8	2.5	2.8	–	–
Blood plasma (steer)	0.35	0.37	0.28	0.27	0.37	0.30	0.33
Fish meal	75.0	79.2	76.0	64.1	62.4	–	–
Chick mash	8.9	10.6	9.1	7.6	8.5		–
Cattle faeces (dried)	1300.0	1260.0	654.0	621.0	668.0	681.0	669.0
Yeast extract (Difco)	5.0	5.0	0.31	0.39	–	–	–

[a]Correction factor of 1.07 was applied to all potency estimates obtained after purification.

plasma, fish meal, or chick mash, indicating that these materials do not contain significant amounts of microbiologically active factors other than cobalamins. The presence of large amounts of other growth-stimulating factors in dried faeces and in yeast extract was suggested by the difference in the values obtained before and after purification. True vitamin B_{12} activity was apparently responsible for approximately 50% of the activity of dried faeces and for less than 10% of the activity of yeast extract. The coefficient of variation of the modified method was 8.7% based on the potency estimates obtained by microbiological assay after purification.

Discussion

The modified procedure appeared to remove pseudovitamin B_{12}, factor A, and desoxyribosides that interfere with the *L. leichmannii* assay. Although small amounts of these factors remained after purification, interference with the assay was negligible owing to the relatively low microbiological activity of these factors. Vitamin B_{12} III, which is very similar in structure to vitamin B_{12} (6, 9) was, however, only partly removed by purification. Vitamin B_{12} III has approximately the same activity for both *L. leichmannii* and *O. malhamensis* (4).

The use of Co^{60}-labelled vitamin B_{12}, as in the original method (1), provided a convenient way of establishing a correction factor for vitamin B_{12} lost during purification. Losses of vitamin B_{12} with the modified procedure were small and relatively constant. This suggested that a single correction factor may be applicable to most natural materials. Although slightly larger losses of vitamin B_{12} may occur during purification of some materials, statistical analysis of repeated tests indicated that, with the possible exception of milk, the correction factor was valid and reproducible. From an analysis of variance there was a suggestion of some bias on the part of individual workers in handling the method. With many materials, a thin solid layer formed at the interface between the solvent phases during purification. Partial removal of this layer during separation of the solvent phases resulted in variable losses of vitamin B_{12}. The bias due to workers appeared to be eliminated by the use of a higher centrifuging speed which compacted the layers and facilitated separation of the solvents.

Metabisulphite extraction of samples was used in place of a cyanide treatment (1) when it was found that bisulphite extraction did not alter the efficiency of purification. Although any one of several microorganisms may be used for the estimation of vitamin B_{12} after purification, *L. leichmannii* appeared to be the most suitable. This organism was found to be less sensitive than *Escherichia coli* 113–3 to traces of organic solvents remaining in the purified extracts.

Comparison of estimates of vitamin B_{12} activity obtained by microbiological assay before and after purification of samples suggested that most materials do not contain significant amounts of microbiologically active factors other than cobalamins. This is in agreement with the findings of other workers (12).

The presence of large amounts of vitamin B_{12}-like factors was undoubtedly responsible for the high activity of ruminant faeces (7) before purification while the activity of unpurified yeast extract was probably due to desoxyribosides.

The modified purification procedure is relatively simple and has been found suitable for routine use. In practice, from six to eight samples can be extracted and purified prior to microbiological assay in about 2 hours by one worker. It is suggested that the method should not be applied to samples known to contain insignificant amounts of interfering materials since the purification process per se undoubtedly increases the total error of the determination. The proposed procedure does, however, provide a convenient method of purifying samples of low potency suspected of containing appreciable amounts of interfering substances, e.g. pseudovitamin B_{12} or desoxyribosides.

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THE DETERMINATION OF THE AVAILABLE CALORIC CONTENT OF RAPESEED OIL BY RAT GROWTH[1]

E. J. Middleton and J. A. Campbell

Abstract

The available metabolic energy of rapeseed oil, semihydrogenated rapeseed oil, and corn oil was measured in terms of 7-day body-weight gains of weanling rats fed a calorically restricted diet. Standard bio-assay procedures were used with lard as a reference standard. The oils were fed at three levels equivalent to 5, 9, and 17% of the diet by weight. Growth responses to the three oils and to lard were similar and the calculated caloric content of the oils showed no significant difference from that of lard. A 1 week assay was as precise as a 5 week assay.

Introduction

The determination of the available caloric content of foods has been based generally on the content of the protein, carbohydrate, and fat multiplied by the appropriate factors. This procedure is indirect and may not give a true estimation of the calories which are physiologically available. It is time-consuming especially if many foodstuffs are to be surveyed.

Oser and Melnick (14) and more recently Rice, Warner, Mone, and Poling (16) and Oser and Oser (15) devised methods of energy measurement on a rat-growth basis. The assumption was that growing animals given an adequate diet but restricted in calories should gain weight in proportion to the amount of calories metabolized. Thus, by relating rat growth to calories supplied, and comparing with an appropriate standard, it was possible to measure the metabolic available energy.

Recently much interest has been shown in the nutritional value of rapeseed oil with a view to its possible incorporation into the human diet. Reports in the literature (7, 3) indicated that rapeseed oil in the diet had a growth-retarding effect. Thomasson (17) reported that body-weight gain decreased as the amount of rapeseed oil in the diet increased and that it was associated with lower food intake. This was confirmed by Beare, Murray, and Campbell (1), who showed that rapeseed oil not only reduced appetite, but in addition had an apparent lower utilization than corn oil as indicated by slower growth over the first 3 weeks of the test. Its gross caloric value, however, as determined by heat of combustion in a bomb calorimeter was known to be practically the same as that of lard (12). Thus it was of interest to have a comparison of the metabolic available energy of rapeseed oil with that of lard.

This paper describes modifications of the rat growth procedure in determining the caloric value of rapeseed oil.

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Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa.

Experimental

A highly nutritious basal diet similar in most respects to that of Rice *et al.* (16) was formulated. The percentage composition of the diet was: casein (vitamin free) 40; corn starch 30; sucrose 15; salt mixture 6 (11); alphacel 4; corn oil 4; vitamin mixture 1. The vitamin mixture made up in alphacel contained per gram, 0.50 mg. thiamine, 1.00 mg. riboflavin, 0.40 mg. pyridoxine, 4.00 mg. calcium pantothenate, 10 mg. niacin, 0.04 mg. vitamin B_{12}, 100 mg. choline chloride, and 80 I.U. vitamin A. Six International Units of vitamin E were added to each 4 g. of corn oil.

Prime steam lard was used as a standard reference source as suggested by Rice *et al.* The rapeseed oil,* from seed of the Argentine variety and containing approximately 40% erucic acid, was alkali refined, bleached, and deodorized. The semihydrogenated oil had a melting point of 42° C. and the hydrogenated oil a melting point of 61° C. and an iodine value of 3.7. The growth test was set up as a multidose assay using three levels of the standard and three levels of the supplement to be tested. The levels were 0.25, 0.50, and 1.00 g. of oil equivalent to 5, 9, and 17% of the diet. These amounts were added to 5 g. of the basal diet and fed to the animals daily.

Weanling male rats of the Wistar Strain were used in all experiments. The animals were given initially 5 g. of basal diet daily for 7 days and weighed. The rats, five per group, were randomized in a block design to remove any bias that might have arisen from position or temperature changes in the animal cage banks. Each block represented animals of similar initial weights. At the end of 7 days, the animals were reweighed. Four experiments were conducted using the 1 week test and one experiment was extended over a period of 5 weeks.

The caloric content of each oil was calculated in terms of the standard by the usual bio-assay procedures as outlined by Bliss (2). Since the doses were equally spaced on the logarithmic scale, it was possible to simplify the calculations by coding the doses.

The feces were collected and pooled for each level of oil for a period of 5 days. Each sample was analyzed for free fatty acids, and neutral lipid material by a 5 hour extraction with petroleum ether in the Goldfisch apparatus. The residue was allowed to stand in petroleum ether containing 5% acetic acid for 2 hours to convert soaps to the free acids. The fatty acids were then removed by extraction in a Soxhlet for 5 hours with the same solvent (10). The animals on the basal diet were used as the control.

Results

The results of a typical assay indicated that there was a linear relationship between the logarithm of the amount of oil and the logarithm of gain in weight of the animals, as illustrated in Fig. 1. It will be noted that the three oils, lard, corn oil, and rapeseed oil, gave similar response curves over the range tested.

*Supplied through the courtesy of the Saskatchewan Wheat Pool and Dr. B. M. Craig, Prairie Regional Laboratory, Saskatoon.

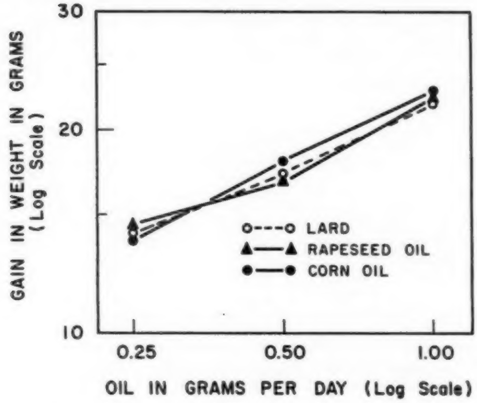

FIG. 1. Rat growth response curves for three levels of lard, rapeseed oil, and corn oil.

Four experiments were carried out with rapeseed oil to determine its caloric content. In Table I, the average weight gain and standard deviation for each level of lard and rapeseed oil for all experiments is shown. The available caloric content of rapeseed oil was found to vary from 7.7 to 10.6 cal. per g. in different experiments. The last test appeared to yield a low result which was significantly different from the results of the other tests.

The available caloric content of four oils and sucrose was determined and the results are shown in Table II. The values were based on lard which was taken to have the generally accepted value of 9 cal. per g. (13). The 95% confidence limits and s/b (standard deviation/slope of response line) values of corn oil, rapeseed oil, semihydrogenated rapeseed oil, and sucrose were within the acceptable range for biological procedures. The very wide limits found in the case of the fully hydrogenated rapeseed oil were a reflection of the difference in caloric value of this sample and of the low slope of the

TABLE I

COMPARISON OF BODY WEIGHT GAINS* OF RATS FED AT THREE LEVELS OF RAPESEED OIL AND LARD

Expt. No.	Supplement	Level of oil 0.25 g.	0.50 g.	1.00 g.	Determined caloric value†
1	Lard	14.5 ± 3.2	17.4 ± 1.7	20.9 ± 2.5	10.6
	Rapeseed oil	14.8 ± 1.3	17.9 ± 2.3	22.7 ± 1.6	
2	Lard	15.2 ± 1.3	19.0 ± 2.3	23.7 ± 1.6	
	Rapeseed oil	17.2 ± 1.5	19.1 ± 1.2	24.0 ± 1.0	10.4
3	Lard	13.1 ± 2.1	14.7 ± 2.0	19.8 ± 1.9	
	Rapeseed oil	12.8 ± 1.5	15.1 ± 1.3	20.9 ± 1.8	9.5
4	Lard	13.4 ± 2.4	17.9 ± 1.2	23.5 ± 2.1	
	Rapeseed oil	13.5 ± 1.3	15.7 ± 1.3	22.4 ± 1.4	7.7

*With standard deviations.
†Lard was the standard with a value of 9 cal. per g.

TABLE II

ESTIMATED CALORIC VALUE OF SEVERAL OILS AND SUCROSE*

	Caloric content			
	Potency determined in calories	95% confidence limits	Coefficient of variation	s/b†
Rapeseed oil	8.9‡	87–116	10.8	0.147
Semihydrogenated rapeseed oil	9.2	82–122	7.8	0.117
Fully hydrogenated rapeseed oil	0.9	53–189	12.2	0.376
Corn oil	8.7	81–124	10.8	0.126
Sucrose	4.0	85–118	8.6	0.096

*Lard was used as standard with caloric content of 9 cal. per g.
†Standard deviation / slope of response line.
‡Mean of four experiments combined by the method of Bliss (2).

TABLE III

EFFECT OF DURATION OF FEEDING ON THE PRECISION OF ESTIMATING THE CALORIC VALUE OF RAPESEED OIL

	Caloric content			
Week	Potency determined in calories	95% confidence limits	Coefficient of variation	s/b*
1	7.7	83–120	10.6	0.120
2	8.2	78–128	10.7	0.141
3	7.4	81–124	11.5	0.126
4	7.3	75–133	14.6	0.166
5	7.0	77–129	12.5	0.144

*Standard deviation / slope of response line.

response line as indicated by the s/b value. The variation as judged by the coefficients of variation appeared to be of the same order as reported by Rice *et al.* (16). Analysis of variance of the data indicated that there were usually no significant differences between blocks of animals representing varying initial weight and position in the bank of cages. Therefore in the calculation of the confidence limits it was not considered necessary to exclude these effects.

In the fourth experiment the animals were weighed weekly for 5 weeks and the caloric content of rapeseed oil calculated for each week. In Table III, the values for the caloric content of rapeseed oil and an indication of the reliability of the determination for each week is given. The results do not differ significantly from week to week either in the estimate of the potency or in the precision.

The coefficients of digestibility were determined for lard, rapeseed oil, semihydrogenated, and fully hydrogenated rapeseed oil at the three levels of supplementation. The results are given in Table IV. The basal diet was used as a control to correct for metabolic fat. Lard, rapeseed oil, and semi-

TABLE IV

COEFFICIENTS OF DIGESTIBILITY OF SEVERAL OILS

Oil	Levels fed		
	0.25 g.	0.50 g.	1.00 g.
Lard	98.9	98.8	98.0
Rapeseed oil	99.1	98.2	97.4
Semihydrogenated rapeseed oil	99.4	98.3	97.3
Fully hydrogenated rapeseed oil	57.4	45.3	34.6

hydrogenated rapeseed oil have digestibility coefficients of 97 to 99% at all three levels. The digestibility of hydrogenated rapeseed oil varied from 35 to 57% and appeared to drop off significantly as the amount of oil in the diet increased.

Discussion

The method described is essentially that of Rice *et al.* (16) with a few modifications. These were (1) lowering the vitamin supplement from 10 to 4 times normal requirement, (2) replacing the destearinized cottonseed oil with corn oil, (3) applying a six dose bio-assay procedure, and (4) calculating the potency on the basis of a standard. Requirements for a valid bio-assay are linearity of response and parallelism between response curves of the standard and the test samples. A linear relationship was demonstrated between body weight gain and the level of oil in the diet on a log basis, and the response curves were parallel. On the basis of a six dose assay with five animals per group the confidence limits at $P = .5$ were approximately $\pm$ 18%. Rice *et al.* found that position in the bank of cages influenced the response to fats. In the present study since blocks represented a combination of position in the banks of cages and variations in initial weight it was not possible to determine the significance of the individual effects. The combined effect, however, was usually below the 5% level of significance.

Rice *et al.* (16) fed levels of lard up to 3 g. per day. In our tests, however, it was impossible to obtain consistent, full consumption at levels above 1 g. fat. Data on the variation of the assay confirm the conclusions of Rice *et al.* and demonstrate no advantage for continuing the test past 1 week. While the caloric content of rapeseed oil appeared to fall off in successive tests, no significance was attached to this since all tests were within the predicted confidence limits except the last one.

Under the condition of restricted feeding imposed in this study, rapeseed oil and semihydrogenated rapeseed oil had the same available caloric content and were not significantly different from lard. This would seem to indicate that the calories derived from the different oils were being utilized equally under the conditions of this test.

The coefficients of digestibility at each level were identical for rapeseed, semihydrogenated rapeseed, and lard. These results agreed with values for

digestibility reported by Holmes (9) and Deuel (8) on humans and unpublished work in this laboratory with rats, but were at variance with the work of Deuel (6) and Carroll (5) on rats. The digestibilities did not fall off with increasing amounts of fat as reported by Rice *et al.* (16). The digestibility of fully hydrogenated rapeseed oil agreed with the caloric value shown for it. In this case, however, the digestibility did decrease with increased amounts of oil in the diet.

On the restricted regimen there was no difference in the rate of growth between animals on lard, corn oil, or rapeseed oil when fed at levels of 5, 9, and 17% of the diet. These findings on a restricted feeding basis differed from those on an ad libitum basis reported by Boer (3), Deuel (6), Thomasson (17), Carroll (4), and Beare *et al.* (1), who found that rapeseed oil retarded growth. This effect was associated with decreased food consumption by Thomasson (17). In addition, Beare *et al.* (1) showed that, when the weight gains were corrected for differences in the amount of food consumed, there was still a significant difference in the corrected gains at 3 weeks indicating that a difference in appetite was not the only effect. There appeared to be two main factors which may have caused the differences between this study and that of Beare *et al.*: method of feeding and protein content of the diet. Further studies involving these differences will be reported in a subsequent paper.

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THE EFFECT OF COLD ACCLIMATION ON THE SIZE OF ORGANS AND TISSUES OF THE RAT, WITH SPECIAL REFERENCE TO MODES OF EXPRESSION OF RESULTS[1]

O. HEROUX AND N. T. GRIDGEMAN

Abstract

In experiments in which two groups of animals of different mean body weight are compared, individual organ weights of the animals can be expressed as absolute weights, as fractional weights, or as absolute weights statistically regressed onto constant body weights. The second, and commonest, mode of expression involves the assumption that the part is directly proportional to the whole, and this is shown to be unlikely for all organs except the muscle mass. Practical as well as theoretical justifications for the use of regressed weights (which utilize the actual slope of the line relating the organ weight to the whole) are given.

The experimental data are from white rats kept for 4 weeks in a warm (30° C.) or a cold (6° C.) environment. It is shown that cold adaptation had no effect on brain, genitals, and lung weights, but that it reduced the growth of muscle, pelt, fat, skeleton, spleen, and thymus, and that it hypertrophied the liver, intestine, kidney, heart, and adrenals. Apparently cold acclimated rats are smaller than the controls mainly because they have a smaller muscle mass.

Introduction

Cold inhibits animal growth as a whole (1, 2, 3, 7, 8, 9), but the effect differs from one organ to another. For instance, several authors (2, 3, 5, 6) have reported an actual *increase* in weight in one or more of the following organs of cold-acclimated animals: liver, heart, kidney, thyroid, and adrenals. The body fat is of course reduced in weight by a cold environment but, as Pagé and Babineau's data show (4), this can account for less than 30% of the total body-weight difference, and we therefore infer that the massive tissues, such as muscle, bone, and pelt, are also reduced.

The present paper is concerned (*a*) with a tissue by tissue investigation into the effect of cold on growing rats, and (*b*) with the expression of the results. In regard to (*b*) we may note that it is often difficult to decide, in work of this kind, whether to use absolute or percentage weights. The endeavor here is to show how different analytical pictures of the same data can be given and to point out the need for careful thought in the choice of the picture most appropriate to the circumstances.

Experimental Conditions

The organs were those of male Sprague Dawley rats maintained for 4 weeks at 30° C. or 6° C. When transferred to the acclimation rooms the rats weighed about 200 g. During acclimation, each rat was weighed once a week. The diet was Master Fox cubes and tap water ad libitum. Individual cages were used.

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa 2, Canada.
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After acclimation the animals were weighed, killed, and dissected without delay. The trunk and the guillotined head were first suspended to drain off the (unmeasured) blood. Then the two parts were flayed, and the pelt, freed of superficial fat tissue, was weighed. Next, the viscera were dissected and weighed individually. The pituitary was included as part of the brain, the thyroid as part of the lungs, and the pancreas, mesentery, and small particles of fat were left adhering to the digestive tract—which was weighed before and after expulsion of contents. All the fat removed from pelt, organs, and carcass was pooled and weighed separately. To obtain the skeletal weight the muscle was digested from the carcass with 10% and 30% aqueous KOH, and the remaining bones were copiously washed in water and weighed after the excess water had been evaporated at room temperature. The wet weight of the whole muscle mass was obtained as the difference between carcass and wet bone weights.

In the tables and diagram, the item "miscellaneous" includes the unmeasured blood, pieces of tissues lost during dissection, and water evaporation.

Theoretical Considerations

Three analytical procedures have been used to represent the intergroup relation of parts. In each, the relation itself will be given in the form

$$(6^\circ\text{ C. part} / 30^\circ\text{ C. part}) \times 100,$$

and the differences will reside in the mode of expression of the weights of the parts. The first mode will be absolute weights, the second fractional weights, and the third absolute weights statistically regressed into constant body weights. The three sets are given in Table III. The derivation of the absolute weights needs no explication, but something must be said about that of the others.

Fractional Versus Regressed Weights

When the weight of organs or other parts of an animal body are studied in relation to the weight of the body itself, the use of fractional weights involves the assumption that, normally, the part is directly proportional to the whole. If the part is large, such as muscle mass, the assumption is satisfactory, but if it is small, the assumption is weak. For instance, it would imply that, in a pair of rats one of which is twice as big as the other, the hearts can be expected to exhibit the same 2:1 ratio. But this violates elementary principles of biological growth. Perhaps the best assumption is that organ and body have a constant differential growth rate, i.e. if Y is organ weight and X is body weight, the allometric equation

$$[1] \qquad Y = cX^b \text{ or } \log Y = a + b\,.\log X$$

holds. In many situations, however, including the present one, in which the range of values is fairly narrow, no information is lost, and computational time is saved, if the logarithmic weights are replaced by the absolute weights in the second, linear form of the equation. This leads to the following

procedure: Estimate the constant b (the value of a is here immaterial) of the relation

$$Y = a + bX \qquad [2]$$

for each homogeneous group of animals, and use it to adjust the group-mean organ weights to allow for differences in group-mean body weights. In effect, we thus partition the observed organ-weight differences into (i) a component associated with body-weight differences, and (ii) another component directly attributable to intergroup treatment differences.

The assumption of proportionality, on which the "fractional" mode of expression is based, can be tested. If it is valid, the expected value of the constant a in equation [2] will be zero, so that the expected slope

$$b_e = \overline{Y}/\overline{X}, \qquad [3]$$

which indicates that the quotient of the mean organ weight and the corresponding mean body weight will be an estimate of the constant b. Now the observed slope, to fit equation [2] without any prior assumption about the value of a, is, by the method of least squares,

$$b_0 = \Sigma xy/\Sigma x^2 = [N.\ \Sigma XY - \Sigma X.\ \Sigma Y] \ / \ [N.\ \Sigma X^2 - (\Sigma X)^2] \qquad [4]$$

where x and y are deviations from the means of the N observed values of X and Y respectively. Therefore, we should expect the quantity b_0/b_e to be unity if the proportionality concept obtains. Slope ratios significantly greater or less than unity will be indicative of non-proportionality of organ weight and body weight, and will thus be pointers to the misleading nature of fractional weights as a mode of presentation.

Another way of looking at the problem is depicted in Fig. 1, in which two compared sets (means and ranges) of hypothetical body and organ weights are shown in six possible regression settings. In graphs 1 and 2, the means lie on regression lines that pass through the origin (i.e. unit slope ratio). In graphs 3 and 4, we have the more likely circumstances: the regression lines do not pass through the origin; the slope ratios are greater than unity. The difference between graphs 1 and 3 on the one hand, and between 2 and 4 on the other, is that the latter show a differential effect on the two organs (they each have specific regression lines, whereas in graphs 1 and 3 the two organs have a common line). In other words, we should infer from graphs 2 and 4, but not from graphs 1 and 3, that the treatment difference has an effect on organ weight per se. Graph 5 is similar to graph 4 except that here the absolute organ weights are identical; nevertheless there is still a treatment difference. Finally, graph 6 represents a more complicated, and probably rare, situation in which the slopes of the two regression lines differ and yet neither passes through the origin.

An analysis of regressed weights, as expounded here, is a means of distinguishing between these various regression settings and is therefore more informative than the simpler methods.

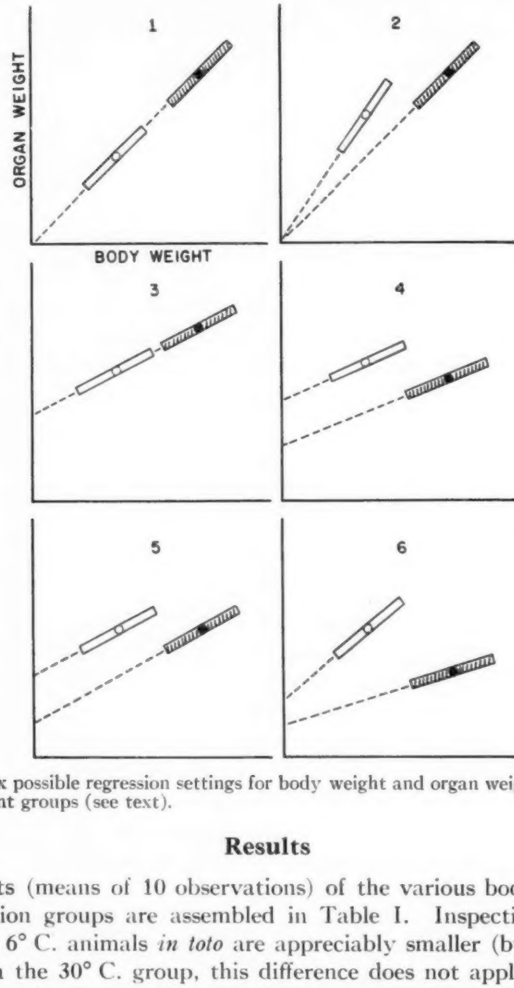

FIG. 1. Six possible regression settings for body weight and organ weight relationship in two different groups (see text).

Results

The weights (means of 10 observations) of the various body parts in the two acclimation groups are assembled in Table I. Inspection shows that although the 6° C. animals *in toto* are appreciably smaller (by almost 20%) than those in the 30° C. group, this difference does not apply uniformly to all the parts. Muscle, pelt, fat, and skeleton are smaller in the colder group, but many of the visceral organs (e.g. intestine, kidney, adrenals) are appreciably larger. Some assurance that this range of differences is not an outcome of normal biological variation may be drawn from the analysis of variance in Table II.

The last column of Table I gives the coefficients of variation (which is the standard deviation of a single observation expressed as a percentage of the mean). We note that the brain seems to be the part that (not surprisingly) varies least from one animal to another, and that spleen and thymus vary most. The pooled coefficient of variation is 16.7 (see third footnote to Table II).

TABLE I

WEIGHTS IN GRAMS OF VARIOUS PARTS OF RATS ACCLIMATED AT 30° AND AT 6° C.
(Each weight is the mean from 10 rats per group)

	30° C. group	6° C. group	Combined coefficient of variation
Muscle	162.04	123.41	7.9
Pelt	49.13	37.49	9.7
Fat	24.09	12.65	17.1
Skeleton	17.36	15.79	10.4
	252.62	189.34	
Brain	1.92	1.87	5.2
Miscellaneous	26.36	26.06	14.3
	28.28	27.93	
Liver	12.274	13.464	11.0
Intestine, etc.	12.177	14.243	7.9
Genitals	7.453	7.135	8.0
Kidney	2.391	2.810	9.0
Lungs	1.655	1.679	11.0
Heart	1.062	1.198	8.3
Spleen	0.885	0.617	30.7
Thymus	0.430	0.320	30.8
Adrenals	0.0392	0.0497	11.7
	38.37	41.51	
Corpse (less intestinal contents)	319.3	258.8	

TABLE II

ANALYSIS OF VARIANCE OF WEIGHTS OF 14 PARTS OF EACH OF 10 RATS IN EACH OF TWO ACCLIMATION GROUPS

	Degrees of freedom (D.F.)	Mean square deviations	Variance ratio (F)
Parts	13	—	
Rats	18	794	
Groups	1	5126	
Interactions:			
Groups by parts	13	3031	16.9
Residual	234	179	1
	279		

NOTE:
(i) For this analysis all parts were initially converted to percentages of the over-all 20-item means.
(ii) The important feature is the $F = 16.9$ for the "groups by parts" interaction, which, being much larger than unity, indicates that the weight distribution of the parts differs significantly in the two groups.
(iii) The residual coefficient of variation is $\sqrt{179} = 16.7\%$, which is the pooled value of the combined coefficients in the last column of Table I.

TABLE III

WEIGHTS OF PARTS (VARIOUSLY EXPRESSED) OF "6° RATS" AS PERCENTAGES OF THOSE OF "30° RATS"

	Slope ratio of organ/body-weight (b_0/b_e)	Absolute weights		Fractional weights		Regressed weights	
		%	t	%	t	%	t
Muscle	0.97	76	7.7	94	3.0	93	1.7
Pelt	1.11	76	6.1	94	2.1	95	0.9
Fat	1.44	52	8.2	65	6.4	69	2.8
Skeleton	0.47	91	2.0	113	2.6	103	0.4
Brain	0.20	98	1.1	120	6.0	103	0.7
Miscellaneous	1.56	99	0.2	122	3.9	138	3.6
Liver	1.17	110	1.9	135	7.5	138	4.2
Intestine, etc.	0.72	117	4.4	144	12.4	134	4.9
Genitals	0.36	96	1.2	118	4.2	104	0.6
Kidney	0.54	118	4.0	145	8.8	132	3.7
Lungs	0.0	101	0.3	125	4.3	101	0.3
Heart	0.54	113	3.2	139	8.9	127	3.5
Spleen	2.54	70	2.6	87	1.2	128	1.2
Thymus	3.05	74	2.1	92	0.7	143	1.7
Adrenals	0.0	117	4.6	156	8.0	117	4.6

NOTE: "t" is the percentage difference in units of its standard error; if $t \geqslant 2$ the difference s considered statistically significant at a probability level of 5%.

Estimates of the slope ratio b_0/b_e, for 13 of the 15 isolated parts of the body are given in the first column of Table III. These estimates were pooled for the two acclimation groups, since they were not significantly different. Two organs, the lungs and the adrenals, showed no correlation with body weight. It is plain that at least some of these non-zero estimates are significantly different from unity, the extremes being the brain at 0.20 and the thymus at 3.05. The standard errors and therefore the precise statistical significances of the estimates could of course be calculated, but here we are concerned only to show that there is practical, as well as theoretical, justification for trying an assumption alternative to that of unit slope ratio.

The alternative to fractional weights, which assume unit slope ratio, is regressed weights, which, as shown above, utilize the internal estimate of the slope. In other words, for each part, we compute the slope b_0 from equation [4] and use it to calculate the equivalent part weight for a standard body weight. This is the basis of the relative regressed weight on the right-hand side of Table III. It is to be noted that the two organs, lungs and adrenals, whose weights are uncorrelated with body weight, do not submit to this procedure, because the regression is zero, so we insert the absolute organ weight instead.

The results are charted in Fig. 2. The column widths have been made roughly proportional to the relative sizes of the parts, which gives the picture an extra dimension.

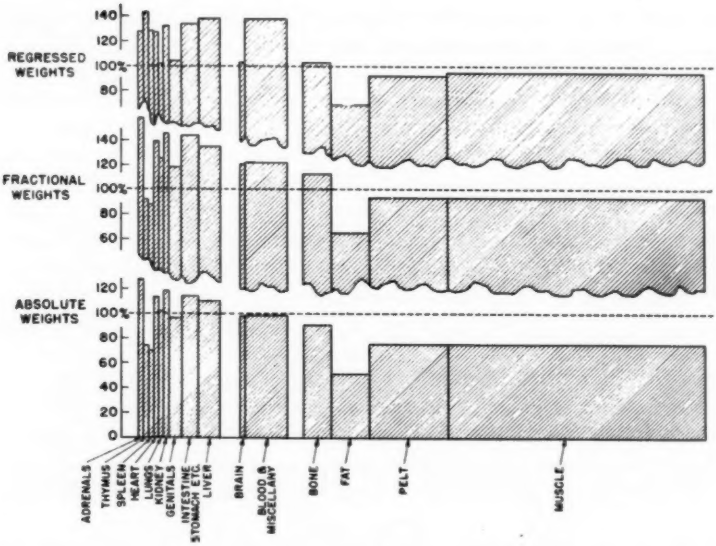

FIG. 2. Showing the weights of parts (variously expressed) of 6° C. acclimated rats as percentages of those of 30° C. acclimated rats.

Discussion

When organ weights are expressed as fractions (percentage weights as usually given in the literature), the situations depicted in graph 1 or 2 of Fig. 1 are assumed. The preceding analysis shows that this situation does not apply to most of the organs since only muscles and perhaps the pelt have regression lines passing through the origin. But, on the other hand, in no case were different absolute slopes found for the two acclimation groups, which thus eliminates situation 6. Therefore only situations 1, 3, 4, and 5 apply to our results; situation 1 to muscle and pelt, and either 3 or 4 or 5 to organs with non-unit slope ratios.

The use of fractional weights will court a particularly prominent error if, as exemplified by the lungs and the adrenals in the present study, the weight of the organ is uncorrelated with body weight. From the fractional weight, which, in the absence of correlation, is virtually meaningless, we should be led to the false conclusion that cold adaptation hypertrophied the lungs. A similar misinterpretation could be reached for the brain and the genitals both of which showed a slight correlation with body weight. In general, the higher the correlation, the lower the danger of misinterpretation.

In studying Table III, we must bear in mind the general rule that when both the absolute weights and the regressed weights are significantly different from 100% (i.e. as between acclimation groups) we can infer a specific effect of acclimation on the organ (graph 4, Fig. 1). Thus, the cold environment

plainly had an atrophying influence on the fat mass and an hypertrophying effect on the liver, heart, intestine, kidney, and adrenals. If the absolute weights, but not the regressed weights, are different (graph 3, Fig. 1) no such direct influence can be assumed. In the present study, for example, the muscles, the pelt, the skeleton, the spleen, and the thymus are smaller in the cold group, but only by virtue of the fact that the whole body is smaller. In this particular situation, because the muscle, the pelt, and the skeleton comprise 75% of the body, the relation can be reversed and we can say that the animals were smaller after cold acclimation because they had a smaller muscle mass, etc. (The emphasis is here on muscle mass itself as it comprised two-thirds of the 75%.) Finally we note that absolute weights alone cannot yield any reliable information on the effect of the experimental treatment per se.

Using the appropriate mode of expressing each organ weight, we can summarize the physiological effect of cold adaptation on individual organ or mass of tissue, as (1) it had no effect whatsoever on brain, genitals, and lungs, (2) it reduced the growth of muscle, pelt, fat, skeleton, spleen, and thymus, and (3) it hypertrophied the liver, intestine, kidney, heart, and adrenals.

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EFFECTS OF SODIUM-FREE MEDIA UPON THE METABOLISM AND THE POTASSIUM AND WATER CONTENTS OF BRAIN SLICES[1]

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Abstract

Brain slices maintain a high concentration of potassium when incubated aerobically in ordinary sodium-containing low-potassium medium but the concentration falls to the level found under plain anaerobic conditions if choline, lithium, or tris-(hydroxymethyl)-aminomethane (tris) are substituted for all the sodium in the medium. With the choline medium, concentration of potassium in the tissue increases markedly if a very little sodium is present; considerably more sodium is required when the main cation present is lithium. A moderately high concentration of potassium is maintained in tissue under anaerobic conditions in sodium-containing medium when glycolysis is stimulated by pyruvate and previous aerobiosis. This high concentration is not found when sodium in the medium is replaced by choline or lithium. The potassium content of slices incubated aerobically or anaerobically with potassium replacing all sodium in the medium is higher than could be accounted for by simple equilibration of the slice fluids with the medium.

The rate of oxygen uptake is not changed when choline or lithium replace all the sodium in the medium; with tris or potassium in place of sodium there is slight inhibition. Anaerobic glycolysis is increased in the choline and potassium media and slightly depressed in lithium medium. The stimulatory effect on anaerobic glycolysis of pyruvate is apparent in the sodium, choline, lithium, and potassium media but the stimulation by a preliminary period of aerobiosis does not occur in the lithium and potassium media.

Under aerobic conditions swelling and "intracellular" (non-sucrose) space is not affected by replacing sodium with choline or lithium. Both are increased in the tris and, especially, in the potassium media. Under anaerobic conditions there is a striking decrease in swelling and intracellular space with the choline medium.

The replacement of a small amount of salt by sucrose causes a decrease in swelling in all media, especially in the potassium medium.

Introduction

In most animal cells the concentration of potassium is higher and that of sodium is lower than in the surrounding fluid. Since there is evidence that both sodium and potassium can penetrate cell boundaries fairly freely (see, for example, Ref. 1) the high intracellular concentration of potassium has been attributed to passive distribution of potassium under the influence of the electrochemical potential gradient set up by an active extrusion of sodium ions from the cells. Recently evidence has been obtained which seems to indicate that the electrolyte distribution may also be affected by active accumulation of potassium in the cell (see, for example, Ref. 2). "Ion pumps" may thus transport sodium out of the cell and potassium into it.

The activity of the "ion pumps" requires energy which is provided by metabolism. This metabolism itself would be affected by the concentrations of sodium and potassium ions in the cell since these cations are known to affect the activity of various enzymic reactions.

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Contribution from the Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute, and the Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec.

Although numerous studies have been made on the changes in metabolism and potassium concentration in brain slices which are brought about by variations in potassium concentration in the suspending medium (see, for example, Ref. 3) the role of sodium has received little attention. In the present work we have studied the potassium content and the respiratory and glycolytic activity of cerebral cortex slices incubated in media which, at least initially, contained no sodium ions.

Methods and Solutions

Adult rats were decapitated, slices of cerebral cortex were prepared, without moistening, and representative samples of the slices were weighed and introduced into Warburg vessels containing the appropriate medium (2 ml. in aerobic, 3 ml. in anaerobic experiments). The vessels were filled with oxygen or nitrogen – 5% carbon dioxide and incubated at 38° C. (For aerobiosis preceding anaerobiosis, oxygen – 5% carbon dioxide was used.) At the end of the experimental period the slices were collected, lightly[2] drained, and weighed again. When potassium was to be determined in the slices they were digested with nitric acid, and the potassium was determined by flame photometry on the digest diluted with 0.2 *M* sodium chloride solution, using a Beckman DU Spectrophotometer with oxyacetylene flame attachment. When sucrose spaces were to be determined, the slices were homogenized in 2 ml. of 6% trichloroacetic acid and sucrose was determined in the centrifuged extract and in the suspending medium by a modification of the method of Hubbard and Loomis (4). Details of all these procedures have been described previously (5, 6).

Rates of oxygen uptake and anaerobic glycolysis were measured by standard manometric procedure.

The media were prepared by mixing the appropriate volumes of isotonic solutions of the various salts. The "normal" medium referred to below for oxygen uptake determinations contained the following in milliatoms or millimoles per liter: Na^+ 159, K^+ 3.6, Mg^{++} 1.2, Cl^- 128, $SO_4^=$ 1.2, PO_4 (mixed) 17.4, glucose 10. The initial pH was about 7.8 but the pH falls to 7.0–7.2 during incubation with tissue. The "normal" medium for anaerobic experiments contained the following: Na^+ 147, K^+ 3.5, Mg^{++} 1.2, Ca^{++} 1.3, Cl^- 128, $SO_4^=$ 1.2, HCO_3^- 24.5, $PO_4^{\equiv}$ 0.4, glucose 10. The sodium-free media were prepared by substituting the relevant salts for the sodium salts in the "normal" media. No sodium was detectable by flame photometry in these media. The salts which were not immediately available were prepared as follows.

Choline bicarbonate was prepared by ion exchange of choline chloride with the resin Amberlite IRA 400 which had been treated with sodium bicarbonate. Lithium, "tris", and choline phosphate buffers were prepared by bringing lithium hydroxide, tris-(hydroxymethyl)-aminomethane, and choline bicarbonate solutions to the required pH with phosphoric acid.

[2]Attempts to drain slices very thoroughly with filter paper resulted in variable, low, non-sucrose spaces. The reason for this is not understood.

When intracellular spaces were to be estimated, usually 1% (30 m*M*) sucrose was substituted for an osmotically equivalent amount (15 m*M*) of the main salt (sodium chloride, choline chloride, etc.) of the medium. The intracellular or "non-sucrose" space was taken as equal to the total weight of the incubated tissue minus the dry weight (19% of the weight of the unincubated tissue) and minus the weight of medium which would contain the same amount of sucrose as was found in the slice (see (5)).

These sodium-free media will be referred to as "choline", "tris", "lithium", and "potassium" media. It should be noted that, except for the "potassium" media, all these media contained potassium in the same concentrations, 3.6 or 3.5 m*M*, as in the "normal" ("sodium") media.

Results

Aerobic Experiments

The results of oxygen uptake and potassium determinations, together with data on swelling and non-sucrose space, are shown in Table I.

In all cases the rates of oxygen uptake were constant during the experimental period. Substitution of choline or lithium for all the sodium of the normal medium had no obvious effect on the oxygen uptake rate; in the tris and potassium media the respiration was active but on the average somewhat decreased.

The potassium content of the slices incubated in the choline, lithium, and tris media fell to the low level which is found when respiration in normal medium is inhibited by the absence of oxygen (6, 7, and see below). The potassium content of slices incubated in the potassium medium was higher than could be accounted for by simple equilibration with the potassium (163 m*M*) in the medium. The average figure was 284 mM. per kg. of fresh weight or

TABLE I

OXYGEN UPTAKE RATES, POTASSIUM CONTENT, SWELLING, AND NON-SUCROSE SPACE OF SLICES OF CEREBRAL CORTEX INCUBATED AEROBICALLY IN NORMAL AND SODIUM-FREE MEDIA

Main cation	Sodium	Choline	Lithium	Tris	Potassium
Oxygen uptake, ml./g./hr.	2.7 ± 0.25 (8)	2.55 ± 0.35 (8)	2.6 ± 0.25 (8)	2.25 ± 0.2 (9)	2.3 ± 0.2 (7)
Potassium, mM./kg. fresh wt.	55 ± 4 (5)	21 ± 1 (4)	15 ± 2 (4)	20 ± 5 (5)	284 ± 21 (3)
Swelling, %					
No sucrose	34 ± 5 (4)	38 ± 6 (4)	40 ± 9 (4)	45 ± 11 (9)	67 ± 9 (3)
Sucrose	29 ± 6 (12)	30 ± 7 (4)	31 ± 1 (4)	40 ± 6 (4)	50 ± 2 (4)
Non-sucrose space, ml./100 g.	32 ± 6 (4)	32.5 ± 10 (4)	29 ± 8 (4)	41 ± 6 (4)	58 ± 6 (4)

NOTE: Averages ± standard deviations (for six or more determinations) or widest difference (for less than 6 determinations).
Numbers of determinations in parenthesis.
Incubation time 60 minutes.
Most of the results were obtained in sets of experiments in which all media were tested on samples of tissue from the same animals. The figures for non-sucrose space were, however, obtained in different, but similar sets, from those in which oxygen uptake and potassium figures were obtained. The figures "Swelling, no sucrose" were obtained in the experiments on oxygen uptake and potassium content; the figures "Swelling, sucrose", were obtained when the medium contained 1% sucrose for the determination of non-sucrose spaces.

197 mM. per kg. of swollen tissue water (final weight less dry weight) and even greater if much of the swelling is considered to be due to uptake of "extraneous" fluid having the same composition as the medium.[3]

Fig. 1 shows the effects, on the potassium content of incubated slices, of varying the amount of sodium which was replaced by choline or by lithium in the medium. About 18 mM. potassium per kg. tissue is held in the slice, perhaps in a "bound" form, when the medium contains no sodium. With choline as the main cation in the medium the potassium content of the tissue was markedly increased by the presence of low concentrations of sodium. When lithium, rather than choline, was the replacing ion, a much greater restoration of sodium in the medium was required before the tissue potassium increased appreciably.

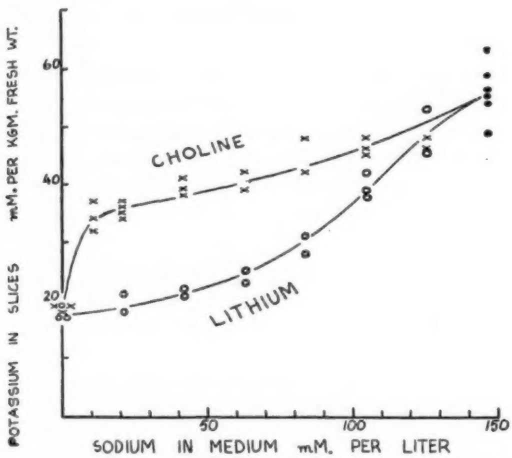

FIG. 1. Effects of varying the sodium concentration in the medium on the potassium content of incubated slices. Aerobic conditions.
× Sodium replaced by choline. ○ Sodium replaced by lithium. ● Normal medium.

The non-sucrose or "intracellular" space was about the same in the choline and lithium media as in the normal sodium medium; it was, on the average, a little increased in the tris medium, and considerably increased in the potassium medium. Such an increase in the intracellular space when the potassium content of the medium is raised has been noted previously (6).

The swelling, that is the increase in weight which always occurs on incubating brain slices in saline media, was about equal in the sodium, choline, and lithium media. It was slightly increased in the tris medium and considerably increased in the potassium medium. In both the latter cases the increased swelling could be approximately accounted for by increases in the non-sucrose or apparent intracellular space.

[3]In the previous study (5, 6) it was concluded that, on incubation, slices take up an amount of fluid, equal to about 40% of the fresh weight of the slices, into a space which is neither intracellular nor normal extracellular; this "extraneous" fluid has the same ionic composition as the medium.

The average swelling which occurred when sucrose was substituted for a fraction of the salt in the medium, in order to determine sucrose space, was always lower than without sucrose. This was true also in the anaerobic experiments mentioned below. The effect in some cases was quite large.

Anaerobic Experiments

Tables II and III show "plain" glycolysis rates, that is rates obtained under simple anaerobic conditions, and rates obtained in the presence of pyruvate, and after a period of aerobiosis and with both these known stimuli. In Table II the rates shown are those measured during the first 20 minutes of readings, after 10 minutes of equilibration under anaerobic conditions. In Table III the rates are those occurring just before the tissue was separated from the medium for potassium or sucrose space determinations.

Substitution of choline for sodium in the normal medium caused a marked increase in glycolysis under all conditions; the stimulatory effects of added pyruvate and previous aerobiosis remained apparent. In the lithium medium there was a distinct depression of glycolysis; previous aerobiosis had no effect but the presence of pyruvate caused stimulation as in the normal medium. When sodium was completely replaced by potassium, plain glycolysis was considerably stimulated. As in the lithium medium, most of the effect of previous aerobiosis was lost though pyruvate caused stimulation. (Anaerobic experiments in the tris medium are not reported because the strong buffer capacity of this medium prevented observation of glycolysis by the manometric method and high absorption of carbon dioxide from the N_2/CO_2 gas mixture complicated the management of the pH.)

It was shown previously (6) that after incubation under simple anaerobic conditions the potassium content of brain slices falls to a low level but, when glycolysis is stimulated by pyruvate or previous aerobiosis, the potassium

TABLE II

ANAEROBIC GLYCOLYSIS BY SLICES OF CEREBRAL CORTEX IN NORMAL AND SODIUM-FREE MEDIA WITH AND WITHOUT PYRUVATE AND PREVIOUS AEROBIOSIS

ml. CO_2 per g. fresh wt. per hr. during the first 30 minutes

Main cation	Sodium	Choline	Lithium	Potassium
Plain	1.30 ± 0.21 *100* (26)	2.19 ± 0.25 *100* (10)	1.06 ± 0.29 *100* (10)	1.76 ± 0.27 *100* (12)
Plus pyruvate	1.96 ± 0.29 *151* (18)	3.54 ± 0.51 *162* (3)	1.70 ± 0.09 *160* (4)	2.31 ± 0.16 *131* (6)
Prev. aerobiosis	2.23 ± 0.39 *172* (18)	3.02 ± 0.21 *138* (3)	1.04 ± 0.22 *99* (8)	1.87 ± 0.23 *106* (6)
Prev. aerobiosis plus pyruvate	3.22 ± 0.59 *248* (20)	4.33 ± 0.52 *198* (3)	1.67 ± 0.31 *158* (10)	2.53 ± 0.10 *144* (6)

NOTE: Averages ± standard deviations (for six or more experiments) or widest difference (for fewer determinations). Numbers of determinations in parenthesis.
Figures in italics indicate relative rates under the different conditions in each medium.
"Plain": simple anaerobic conditions. "Plus pyruvate": pyruvate, 2 m*M* added to the medium. "Prev. aerobiosis": O_2/CO_2 in vessels for 25 minutes; then gas changed to N_2/CO_2. "Prev. aerobiosis plus pyruvate": same, with pyruvate added after the aerobic period.

content of the slice is kept considerably higher. In Table III it is shown that this improved maintenance of tissue potassium does not occur when sodium in the medium is replaced by choline or lithium.

As under aerobic conditions, the potassium content of slices which had been incubated in the potassium medium was higher than could be accounted for by simple equilibrium with the medium (151 m*M* potassium). The average figure after simple anaerobiosis was 271 mM. per kg. of fresh weight or 179 mM. per kg. of swollen tissue water. After previous aerobiosis and with pyruvate the corresponding figures were 307 and 177.

TABLE III

GLYCOLYSIS RATES, POTASSIUM CONTENT, SWELLING, AND NON-SUCROSE SPACE OF SLICES OF CEREBRAL CORTEX INCUBATED ANAEROBICALLY IN NORMAL AND SODIUM-FREE MEDIA

	Sodium		Choline		Lithium		Potassium	
Main cation	Plain	Stimulated	Plain	Stimulated	Plain	Stimulated	Plain	Stimulated
Glycolysis, $ml.CO_2/g./hr.$ Last 10 min.	0.7 ±0.2 (26)	2.5 ±0.45 (20)	1.8 ±0.2 (10)	3.4 ±0.45 (6)	0.5 ±0.15 (10)	1.3 ±0.25 (10)	1.25 ±0.3 (12)	2.0 ±0.15 (6)
Potassium, mM./kg. fresh wt.	19 ±2 (14)	32 ±3 (14)	17 ±2 (4)	16 ±2 (4)	16 ±3 (4)	15 ±2 (4)	271 ±19 (4)	307 ±9 (4)
Swelling, %								
No sucrose	59 ± 8 (16)	55 ± 7 (15)	38 ± 7 (4)	39 ± 7 (4)	53 ± 9 (4)	57 ± 12 (4)	70 ± 5 (6)	93 ± 5 (6)
Sucrose	49 ± 6 (6)	36 ± 5 (6)	30 ± 2 (2)	36 ± 2 (6)	44 ± 8 (4)	40 ± 8 (10)	52 ± 2 (6)	48 ± 2 (4)
Non-sucrose space, ml./100 g.								
60 min.	45 ± 6 (6)		17 ± 1 (2)		48 ± 2 (4)		54 ± 8 (6)	
90 min.	46 ± 6 (6)	43 ± 5 (6)	19 ± 2 (2)	25 ± 6 (6)	37 ± 5 (8)	26 ± 5 (10)	52 ± 4 (4)	48 ± 2 (4)

NOTE: Averages ± standard deviations (for six or more determinations) or widest difference (for less than six determinations).
"Plain": After 60 minutes anaerobiosis, except that figures obtained after 90 minutes are also given for non-sucrose space.
"Stimulated". O_2/CO_2 in vessels for 25 minutes then gas changed to N_2/CO_2, pyruvate, 2 m*M* added and run anaerobic for 60 minutes.
See footnote to Table I for other details.

The non-sucrose space increased under simple anaerobic conditions in the normal medium as previously reported (5). In the lithium medium, also, this space became greater than under aerobic conditions. In the potassium medium, as mentioned earlier, this space became enlarged under aerobic conditions; no further increase occurred under anaerobic conditions. In the choline medium, on the other hand, there was a distinct decrease in this space under anaerobic conditions. The non-sucrose space showed no obvious change when the incubation time in the various media was increased except in the case of the lithium medium. In the latter medium the space decreased on longer incubation, which suggests that a slow penetration of sucrose through membranes was occurring. When glycolysis was stimulated the non-sucrose space increased in the choline medium and decreased in the lithium medium.

As previously reported (5) brain slices in normal medium swell more under anaerobic than under aerobic conditions; this occurred also in the lithium medium. Extra swelling was not apparent in the choline medium and this is partly accounted for by the decrease in intracellular space in this medium.

The large swelling in the potassium medium was about the same as under aerobic conditions. (The very high figure under "Potassium stimulated" in Table III is presumably due to the extra total incubation time—90 instead of 60 minutes.)

Discussion

It is recognized that in brain slices many, perhaps nearly all, cells have been damaged by the cutting of processes of neurones and glial cells. The physiological status of the cells in brain slices is unknown but metabolically they are active and their ability to concentrate potassium is evidence that the basis for a resting potential is at least partially preserved. Study of this type of preparation can therefore help in the understanding of electrolyte distribution in a tissue which is not readily amenable to more rigorous investigation of this problem.

Only brain slices that are actively producing energy contain high concentrations of potassium after incubation in a saline medium containing the normal low concentration of potassium. This energy can be produced by oxidation (7) or, less effectively, by glycolysis which has been stimulated by pyruvate or previous aerobiosis (6). It has now been shown that although oxidative metabolism in brain slices proceeds actively in saline media which contain either sodium, choline, lithium, or tris(hydroxymethyl)-aminomethane as the main cation, a high potassium concentration is found in the incubated slices only when sodium is present in the medium. Anaerobically too an increased potassium content was found, when glycolysis was stimulated, in the sodium medium but not in the cnoline or lithium media. In the case of the choline medium this is true in spite of extra-active glycolysis. The ability to concentrate potassium in the tissue is thus specifically dependent on the presence of sodium in the medium.

The estimates of the "intracellular"[4] (non-sucrose) space indicated that, under aerobic conditions, failure to maintain a high potassium concentration in the tissue cannot, apparently, be ascribed to a disappearance of the space in which potassium is usually believed to be concentrated. Since under anaerobic conditions in which glycolysis was stimulated the apparent intracellular space found in the choline and lithium media was smaller than in the sodium medium, it is possible that the concentration of potassium in the tissue in these cases is partly limited by the volume of intracellular fluid.

Presumably the high concentration of potassium in the tissue is mainly the result of the activity of a "pump" mechanism which extrudes sodium and accumulates potassium within cells or within subcellular structures. It might

[4]It should be emphasized that discussion of intracellular and extracellular spaces in brain tissue involves concepts about which our ideas are far from clear. In previous work (5) it was shown that, in slices of cerebral cortex incubated aerobically, the non-sucrose and non-thiocyanate spaces are equal to each other and to the non-chloride space calculated from figures given by Leaf (8). Inulin apparently penetrates only some abnormal space in the swollen tissue and the non-inulin space is nearly equal to the total water of unincubated tissue. Electron microscopy (see, for example, Ref. 9) has shown that nervous tissue is occupied by a dense multiplicity of structures and provides no indication as to which membranes separate an intra- from and extra-cellular space.

be noted that, in sodium-free media, the potassium content of the tissue was not appreciably higher under aerobic conditions, when oxidative energy was available, than under anaerobic conditions. There was thus no sign of an inward potassium pump which could act in the absence of sodium. The present results could be explained by assuming that the active extrusion of sodium allows potassium to accumulate in the tissue and that only sodium, and none of the other monovalent cations tested, can be extruded. Another possibility is that the accumulation of potassium is dependent upon the provision of energy in the required form, presumably as high energy phosphate, and this provision is inadequate when sodium is absent. Studies intended to test the second possibility are in progress.

The results illustrated in Fig. 1 suggest that both choline and lithium penetrate cell structures and are not "pumped out" like sodium. But, while choline does not interfere with the pumping of sodium, lithium inhibits this activity and an increased sodium, or reduced lithium, concentration is necessary before the activity of the pump mechanism and consequent increase in tissue potassium become apparent.

When all sodium in the medium is replaced by potassium, the concentration of the potassium in the slices becomes remarkably high, aerobically or anaerobically. This accords with our previous conclusion (6) that a considerable fraction of the potassium found in tissue is in a bound form and that the amount present in this form increases with increasing potassium concentration in the medium.

It is well known (10, 11) that addition of extra potassium chloride to the usual sodium-containing medium causes a marked, though variable and transitory, increase in oxygen uptake and aerobic glycolysis and an inhibition of anaerobic glycolysis. Inhibition of anaerobic glycolysis has also been observed when potassium was substituted for part of the sodium in the medium (12). It was therefore somewhat surprising to find that, when sodium was completely replaced by potassium, the oxygen uptake was slightly decreased and "plain" anaerobic glycolysis was increased. These different effects are difficult to explain though the stimulation of glycolysis in the choline and potassium media may be due to the exclusion of sodium which is known (13, 14) to inhibit glycolysis by supplemented tissue extracts. Glycolysis is inhibited in the lithium medium, which suggests that lithium is more inhibitory to glycolysis than sodium.

Rosenthal *et al.* (15) believed that the stimulation of anaerobic glycolysis by previous aerobiosis and by pyruvate in liver slices occurred through different mechanisms. Elliott and Henry (16) found that, with brain suspensions, the effect of previous aerobiosis could be accounted for as an effect of the traces of pyruvate which are produced and maintained under aerobic conditions. The results given in Table II, however, show that with brain slices the mechanisms are distinct since pyruvate exerts its stimulatory effect in all the media while the effect of previous aerobiosis occurs in the sodium and choline media but is absent or much diminished in the lithium and potassium media. In another

communication (in preparation by Rosenfeld, Johnson, and Elliott) conditions will be described in which previous aerobiosis causes stimulation but pyruvate produces none. The basis for the loss of the previous aerobiosis effect in the lithium and potassium media, and for the differences between the cations in this respect, is not clear. The possibility is being tested that it may be related to the extent to which the tissue content of high energy phosphate is affected by the different ions.

The variety of effects of the different media on swelling and non-sucrose space, particularly under anaerobic conditions, cannot be interpreted at present. Presumably slow penetration of sucrose through membranes under some conditions and effects of energy metabolism on these membranes are concerned in these confusing effects.

The effect of sucrose introduced for determination of sucrose space is also noteworthy. This solute, added in low concentration and without altering the osmolarity of the medium, always decreased the total swelling, and this effect was specially marked in the potassium medium. Elliott (17) found that the effects of sugars on the swelling of brain slices was potentiated by the presence of electrolytes. It is evident that this potentiation is dependent upon the nature of the particular electrolyte.

No two of the monovalent cations studied, sodium, potassium, choline, lithium, and tris, have been found to affect the metabolism, potassium content, and water distribution in brain tissue in exactly the same way. It is apparent that each ion has some sort of specific effect and probably none can be regarded as behaving in the tissue as a simple particle with no properties other than that due to its charge. Comparable studies on the effects of anions have not been made. Thomas and McIlwain (18) have observed no obvious change in respiration or aerobic glycolysis of guinea pig cortex slices when sulphate or nitrate was substituted for chloride; with acetate, respiration decreased with time and aerobic glycolysis was high.

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MELANIN PIGMENTATION IN BOVINE PINEAL GLAND AND ITS POSSIBLE CORRELATION WITH GONADAL FUNCTION[1]

ENRIQUE SANTAMARINA

Abstract

The incidence of melanin in the bovine pineal gland was studied in 880 glands. Spectrophotometric analysis and chemical and cytochemical methods identified the black pigment present in the bovine pineal gland as melanin. No melanin was found in bulls 4 and 5 years of age nor in heifers of about 18 months. In pregnant cows over 5 years of age melanin was found in 5.4% of the pineal glands. Non-pregnant cows of the same age exhibited melanin in 8.5% of the pineal glands. Castrated male cattle between 18 and 24 months of age showed 49.6% of the pineal glands with macroscopical signs of melanization. As much as 67% of the pineal glands of steers from some herds contained melanin. In intact cattle melanin in the pineal appears to be mainly an aging phenomenon. The fact that castration in male cattle causes hypertrophy of the pineal gland followed by a degenerative process in which melanin is involved seems to give strong evidence of a pineal gonadal interrelationship. The possible role of the hormones in the phenomenon of melanin formation is discussed.

Introduction

Since the first conference on the biology of normal and atypical pigment cell growth (5), copious literature on melanin has been published. However, studies on the presence of melanin in the pineal gland have not been reported since Jordan (23, 24) described melanic granules in the pineal gland of sheep and Rio-Hortega (41–44), who studied pigmented granules in the parenchymatous cells of the adult human pineal gland, found pigment granules also in pineal glands from other mammals. Santamarina and Venzke (48) reported that adult cattle presented a common phenomenon of melanin infiltration in the pineal gland. More recently Santamarina and Meyer-Arendt (49) published the chemical, histochemical, and spectrophotometric methods used for the identification of pineal melanin.

The present paper discusses the methods used for the identification of pineal melanin, the incidence of melanin in the bovine pineal gland, and the possible physiological relationship of gonadal function to pineal melanin.

Materials and Methods

A total of 880 bovine pineal glands from Guernsey, Ayrshire, Jersey, Holstein, and Brown Swiss breeds were collected mainly in a Columbus, Ohio, packing house. The age of the animals was determined by the dental pattern (50, 55). The glands were collected about 15 minutes after the animals were slaughtered, and those to be used for histological and histochemical studies were put into the corresponding fixative fluids after the capsular membrane was cut in several places to allow for a more rapid penetration of the fixative.

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Contribution as a paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Department of Dairy Industry, New Brunswick, New Jersey. This investigation was initiated while the author was the recipient of a postdoctoral scholarship granted by the graduate school of Ohio State University, and was completed at Rutgers University, in the Department of Dairy Industry, New Jersey Agricultural Experiment Station, with the support of grant RG-4726 from the National Institutes of Health, United States Public Health Service.

Chemical Tests

With the aid of a binocular loop, 100 mg. of black tissue was removed from two pineal glands. This tissue was ground and homogenized in a 40-ml. Potter–Elvehjem grinder with 40 ml. of distilled water.

(*a*) Ferric chloride test: Twenty milliliters of the pineal homogenate was put into a 50-ml. test tube with 1 ml. of a 5% aqueous solution of ferric chloride. This solution gave a gray precipitate which gradually blackened when left standing at room temperature.

(*b*) Bromine water test: Twenty milliliters of pineal homogenate was put into a 50-ml. test tube to which 20 ml. of bromine water was added. The solution, left standing at room temperature, gradually became darker in color and produced a yellow precipitate which finally turned black.

Spectrophotometric Techniques

With the aid of a binocular loop, all the black tissue was obtained from a bovine pineal gland. The same amount of tissue was also taken from a non-pigmented gland. The pigmented and non-pigmented tissues were ground separately and homogenized in a 40-ml. Potter–Elvehjem grinder with 20 ml. of 5% KOH and then kept for 48 hours in a refrigerator where they were agitated frequently. The turbid solutions were centrifuged for 20 minutes at 2000 r.p.m. Four milliliters of the supernatant was pipetted from the middle of each of the tubes and placed in two Beckman rectangular cells. The solution taken from the non-pigmented tissue served as a blank. The solutions used for ultraviolet measurements were further diluted with KOH (1:10) and placed in silica cells. The spectrophotometric measurements were taken with a Beckman DU spectrophotometer.

Histochemical Techniques

The silver method, safranin staining, and bleaching reactions were performed following the procedure described elsewhere (49). Other methods used in the identification of melanin were Dublin's application of the Bodian method (17) and Masson's method (29). Other sections were stained with hematoxylin and eosin; deparaffined unstained sections were also studied.

Results

Chemical Analysis

The ferric chloride and the bromine water tests for melanin gave positive reactions in glands with macroscopically visible black pigmentation. However, these tests failed to detect melanin in glands in which melanin was identified by cytochemical and spectrophotometric methods.

Spectrophotometric Analysis

Visible-light Absorption Spectrum

This method of identification of melanin in pineal potassium hydroxide extracts proved to be very accurate. Glands which macroscopically showed no pigmentation gave the characteristic absorption spectrum of melanin under spectrophotometric analysis.

Fig. 1 shows the characteristic spectral absorption of pineal melanin extracted with 5% KOH. The upper curve represents the absorption spectrum of a centrifuged supernatant obtained from a pineal gland soon after centrifugation. The lower curve, which represents the absorption spectrum of the same solution after 24 hours of standing in the refrigerator, shows a decrease in absorption values typical of melanins kept standing in solutions of high pH. The visible-light absorption spectrum of pineal melanin displayed a strong maximum close to the ultraviolet band, and decreased greatly in the red band of the spectrum without showing any absorption peak.

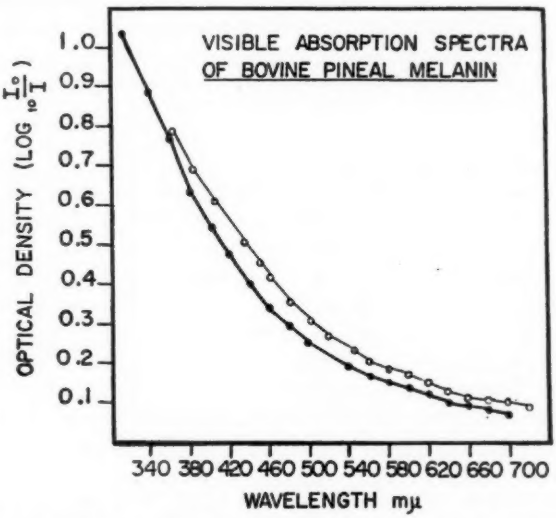


FIG. 1. Curves obtained with a Beckman spectrophotometer, showing the visible light absorption spectra of melanin extracted from one pineal. The upper curve was obtained from a solution centrifuged after 48 hours of extraction. The lower curve shows a decrease in absorption spectra of the same solution after another 24 hours.

Ultraviolet Absorption Spectrum

The same solutions used for visible-light spectral studies were used for ultraviolet studies after further dilutions were made. The curves obtained from pineal melanin exhibited characteristics similar to those obtained from other natural and synthetic melanins.

Fig. 2 shows the absorption spectra of two bovine pineal glands: the upper curve represents the ultraviolet spectrum of the solution studied in Fig. 1; the lower curve shows the ultraviolet spectrum of melanin extracted from a gland which macroscopically showed no pigmentation.

Macroscopical Observations

The data obtained from the macroscopical observation of 880 bovine pineal glands showed that melanin is often present in the bovine pineal in animals of both sexes, in all the breeds studied, and in old and young animals (Table I).

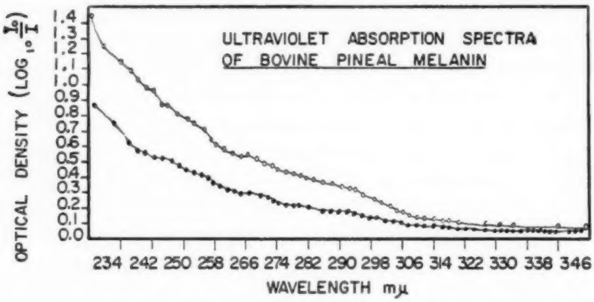


FIG. 2. Spectral curves of melanin obtained from two pineal glands. The upper curve shows the ultraviolet absorption spectra of the solution used in obtaining the curves in Fig. 1. The lower curve was obtained from melanin extracted from a "non-pigmented" gland.

No macroscopical signs of melanin were found in bulls of 4 and 5 years of age. In the steer group 183 out of 369 glands (49.6%) exhibited intense melanization, but although animals from one herd might show a very high percentage of glands with melanin, those of another would show a considerably lower percentage, so that the range among all the groups of steers ran from 38 to 67% of glands with melanin. Thirty-one heifers with an average age of 18 months showed no melanin pigment in their pineal glands. Thirty-seven pregnant cows, most of them between the second and fourth months of pregnancy, showed two glands with melanin. The non-pregnant group of cows, all with functional ovaries, showed 35 glands out of 409 with melanin. No correlation was found between breed pigmentation and melanization of the pineal gland.

Although the pigment seems to appear more often in the free end of the pineal gland first, black spots can be found in any part of the gland. The process of melanization spreads not from a single spot but rather from various points and in such a way that the total melanization is the result of the confluence of many centers of pigmentation. Pigmentation seems to take place

TABLE I

INCIDENCE OF MELANIN IN BOVINE PINEAL GLANDS
(macroscopical observation)

Animals	Estimated age	Total no. glands	No. glands with melanin	Percentage of glands with melanin
Bulls	4 years	8	0	0
Bulls	5 years	26	0	0
Steers	18–24 months	369	183	49.6
Heifers	16–20 months	31	0	0
Pregnant cows	Over 5 years	37	2	5.4
Non-pregnant cows*	Over 5 years	409	35	8.5

*All the animals in the non-pregnant group exhibited functional ovaries.

in apparently hypertrophied glands, that is, large glands which exhibit a high vascularization. The first sign of atrophy almost always appears in the free end of the gland. In cases of intense pigmentation most of the glands showed a very poor vascularization. Of some glands only a slender, black, cylindrical, atrophied organ remained. The average weight of the pineal gland in intact adult cattle was 324 mg., ranging from 184 to 517 mg.

Histochemical and Histological Features

Sections of pigmented pineal gland treated with silver nitrate showed melanin granules in the pigmented areas of the capsular connective tissue, pia mater (Figs. 3, 4). By grouping together, these rounded, dark-brownish granules gave the appearance of masses of melanin granules (Figs. 5, 6). The pigment was arranged mainly around blood vessels of various sizes (Fig. 5), and in many instances the melanin infiltrated the entire wall of the blood vessel (Figs. 7, 8). The *tunica adventitia* of many vessels was often covered by masses of melanin (Fig. 7). In heavily pigmented areas the pigment penetrated through the endothelial cells into the blood vessels, not as regularly shaped granules, but rather as clusters of melanin (Fig. 8).

In the bovine pineal gland the connective tissue – vascular cords form an extensive network which gives a pseudoalveolar architecture to this organ (Figs. 3, 4). As a result of this pseudoalveolar structure the parenchyma of the bovine pineal gland constitutes an almost continuous framework of anastomosed parenchyma and neuroglia cells. The supposedly main secretory elements of the pineal gland, the parenchyma cells, also called pineal or specific cells, are melanin-bearing cells. They showed melanic cytoplasmatic granules in pigmented glands. These cells with melanin, few in number, exhibited fine granules in small or large amounts; in cases of small amounts of granules, the pigment appeared over the distal poles of the nuclei. In some cells a line of single granules extended throughout the long processes of the parenchymatous cells. Other cells were laden with melanin (Fig. 6), and the granules formed a nuclear cap, or completely surrounded the nucleus. Some cells contained such enormous amounts of melanin that even their processes were filled with pigmented granules. The intersection of these pigment-laden processes far away from the nucleus gave the impression of intraprotoplasmatic clumps of melanin.

The number of pineal cells bearing melanin was not in proportion to the degree of melanization of the glandular stroma. Single pineal cells with melanin were rarely found; instead, several cells in the same area showed melanin granules. These groups of pigmented cells were sometimes widely separated.

The signs of degeneration observed in the highly melanized bovine pineal glands were large clumps of melanin granules, the infiltration of melanin in the endothelium of the arterioles, the occlusion of the lumen of some blood vessels, the increase of the glial areas, and an increase in glandular connective tissue.

Discussion

The lack of precise knowledge about the exact nature of pineal pigment resulted in discordant interpretations of the possible meaning of these protoplasmatic granules. Dimitrova's description of some secretory granules in the pineal gland of Bos Taurus (12) corresponded, undoubtedly, to the pigmented granules studied by Jordan in sheep (23, 24), Rio-Hortega in other mammals (42, 43), and the melanin granules described in this study. Although Jordan and Rio-Hortega refer often to the pineal pigment as melanin, neither author used specific methods of identification for this pigment. Since silver methods for the identification of melanin yield varying results (35), cytochemical criteria must be corroborated by chemical and spectrophotometric analyses.

The visible and ultraviolet absorption spectra of pineal melanin showed spectral characteristics similar to those of natural and synthetic melanins (34, 9, 7, 4, 56, 37). Since the visible-light spectrum of pineal melanin was discussed elsewhere (49), mention need only be made here of the fact that the ultraviolet spectral curves of bovine pineal melanin showed the same characteristics as the curves obtained from tyrosine melanin (9) and from a synthetic melanin prepared from tyrosine and potato tyrosinase (56). However, pineal melanin differs slightly spectrophotometrically from the melanin obtained from the choroid of the ox eye (56).

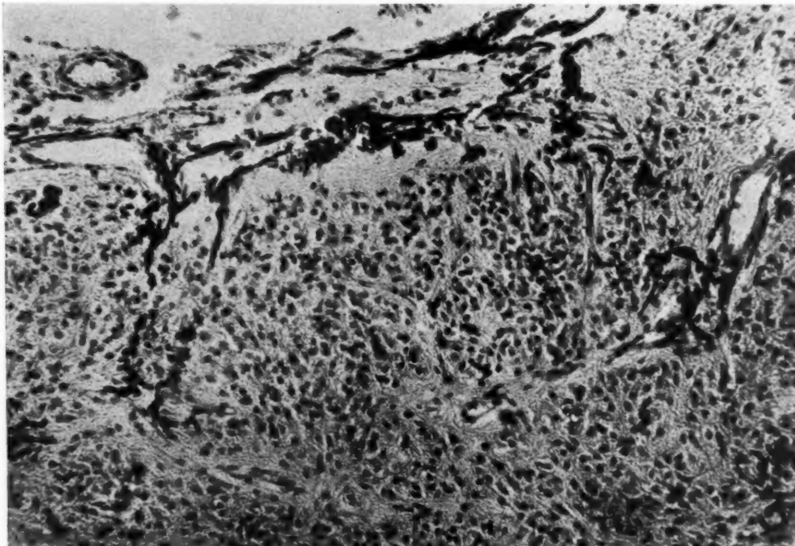
Two main factors may account for the presence of melanin in the pineal gland: (*a*) the histological structure of the gland and (*b*) the hormonal interrelationship of the pineal with other glands of the endocrine system.

The histological structures which favor the phenomenon of melanization of the pineal are (*a*) the encapsulation of the gland by pia mater; (*b*) the pseudoalveolar structure formed by the connective tissue which arises from the capsular pia mater; and (*c*) the high vascularization of the organ. Aschoff (3) was of the opinion that epithelial, endothelial, and connective tissue cells could secrete melanin; Kaufmann (26) found melanin in the deeper layers of the pia, and Goldberg (18) observed melanosis of the spinal meninges and adventitia of the blood vessels in a calf. Schumacher (51) observed melanin precursors and cells with finite melanin in small vessels in the pulpa of the teeth.

Although the histological components of the pineal are capable of producing melanin, the factors inducing melanization are presumably enzymatic and hormonal.

Administration of pineal substances to tadpole (60, 21, 36, 1, 6), toad (6), and fish (14, 64) produces alterations in pigmentation. The role of the pineal in the control of pigment cells in recent teleost fishes has also been investigated (8). However, the fact that the pineal gland itself contains an active substance capable of inducing changes in pigmentation, and that it also has a histological structure suitable for melanin formation, are only two of the many factors known to be involved in pigmentation.

PLATE I



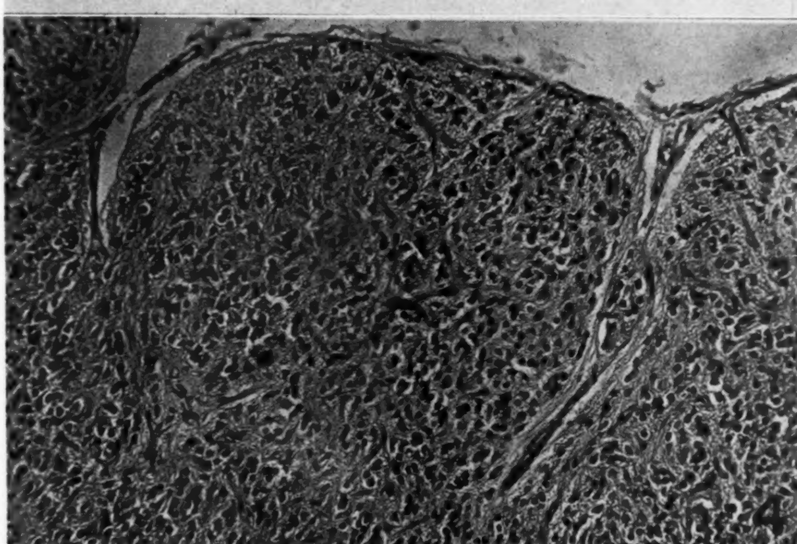


FIG. 3. Photomicrograph of a bovine pineal gland showing melanin infiltration from the capsule deep into the glandular stroma. Sect. 8 micra, × 120.

FIG. 4. Photomicrograph of a bleached section, from the same gland, although different area. Sect. 8 micra, × 120.

The role of androgens in developing melanin has been studied in castrated and eunuchoid man (19, 13), ground squirrel (62), and hamster (30, 20). Since pineal melanosis is a common phenomenon in steers and rare in adult normal males, one may assume that androgens are not primarily involved in the process of melanin formation in the pineal gland of the steer.

Other hormones besides MSH (melanocyte-stimulating hormone) and androgens are also involved in the process of melanin formation, for example, adrenalin (61), noradrenalin (31), cortisone and hydrocortisone (31), adrenocorticotrophic hormones (58, 59, 22, 31); the reader may also be referred to other studies on melanin pigmentation (10, 11, 32, 39, 45, 63).

Castration may affect pigmentation of the pineal in many ways, perhaps by inhibiting or stimulating the production of any of the above-mentioned hormones or by increasing the capacity of the pineal gland to form melanin. The data accumulated in the past 5 years point, in one way or another, towards a relationship among pineal gland, gonads, and pituitary gland (38, 54, 53, 57, 46, 47, 33, 52, 27, 28, 2, 15, 16, 25, 40). This endocrine interrelationship may well explain the pathological phenomenon which occurs in the pineal gland after castration. In most animals, after the removal of the target organ, the gland which stimulates it undergoes a process of hypertrophy. In this case, the removal of the testicle causes the hypertrophy of the pineal gland. During this period of pineal hypertrophy this gland is under the influence of another hypertrophied gland, the pituitary, which releases MSH in sufficient amounts to stimulate melanin production in the pineal gland, which in normal young animals seems to be somewhat refractory to melanocyte-stimulating hormone. Also, the hypertrophied gland may be more sensitive to the nervous stimuli involved in melanization.

The beginning of melanization in steers usually appeared in hypertrophied glands, and in advanced degrees of melanization the melanin filled the small blood vessels, an obstruction that originated degenerative areas. In the early stages of the process, the glands from castrated animals showed a histological picture similar to that of the melanization caused by age in the pituitary and adrenals. It seems that the process of hypertrophy produced in the pineal gland by castration exhausts the gland far more within a short period of time than the exhaustion produced in the span of life. Whatever the intimate mechanism of melanin formation in castrated bovine pineal gland, this phenomenon seems to give strong evidence of a relationship between the pineal gland and the gonads.

Acknowledgment

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ACETATE UTILIZATION BY LIVER AND ADIPOSE TISSUE OF RATS FASTED IN THE COLD[1]

WILLIAM F. PERRY

Abstract

The *in vitro* incorporation of 1-C^{14} and 2-C^{14} acetate into fatty acids and carbon dioxide by liver and adipose tissue was studied in rats fasted at 5° C. for 24 hours. Compared with fed rats at room temperature, there was a marked decrease in the incorporation of the acetate carbons into fatty acids and carbon dioxide by liver tissue. A pronounced decrease in acetate incorporation into fatty acid was also noted with adipose tissue from these same animals, but only a slight decrease in incorporation into carbon dioxide. Addition of glucose to the incubation medium caused increases in fatty acid formation by liver and adipose tissue from both normal and fasted animals, but glucose supplementation, while increasing the incorporation of acetate into carbon dioxide by liver tissue from cold fasted rats, did not affect carbon dioxide production by liver tissue from normal animals. Incorporation of acetate into carbon dioxide by adipose tissue was unaffected by glucose supplementation with tissue from both normal and cold fasted rats.

Introduction

The intermediary metabolism of fatty acids is a complex process which is modified by a number of hormonal, nutritional, and environmental factors. Study of fat metabolism by varying such conditions can further the understanding of the process of fat synthesis and utilization. It has been shown, for example, that either fasting (1,2) or acute exposure to cold (3) leads to a marked diminution in the incorporation of the carbon atoms of glucose or acetate into fatty acids by liver tissue. Recently Masoro *et al.* (4,5) reported that liver slices from rats fasted at either room temperature or at 0–2° C. for 24 hours, when compared with slices from normal non-fasting rats, showed a marked impairment in the incorporation of the labelled carbon of 1-C^{14} acetate into fatty acids and carbon dioxide; but that when such slices from fasted animals were incubated in a medium to which glucose or pyruvate had been added, these defects were corrected wholly or in part. Addition of glucose to a medium containing liver slices from fed animals did not stimulate carbon dioxide production from acetate but did enhance acetate incorporation into liver fatty acids.

These findings are not entirely in agreement with earlier reports by other workers (1,2,6) which showed that liver slices from fasted animals, though defective in lipogenesis, had no impairment in ability to convert acetate to carbon dioxide.

The purpose of the present work was firstly to reinvestigate this phenomenon in view of these contradictory reports and also to see if similar results would be obtained with 2-C^{14} acetate; and secondly to study the effect of fasting on acetate utilization by adipose tissue to observe whether the *in vitro* addition of glucose had similar effects to those reported for liver tissue. This aspect

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of the study was undertaken because alterations in the metabolism of acetate or glucose by liver tissue are not necessarily parallelled by like changes in adipose tissue (7), and because Milstein and Hausberger (8) have reported that adipose tissue, unlike liver, suffers little diminution in its ability to utilize carbohydrate in the presence of a falling blood sugar.

Methods

Wistar strain adult rats of both sexes were fasted for 24 hours at 5° C.; control animals were fed a normal diet ad libitum and kept at room temperature. Both groups were allowed free access to water. The animals were killed by decapitation; the liver and intestinal mesentery, which was used as a source of adipose tissue, were removed; slices were prepared and incubated in a buffered medium at 37° C. for 3 hours with 2 μc. of C^{14} acetate labelled on either carbon atoms 1 or 2 (total acetate concentration in medium 1.5 μM./ml.). The medium for liver was a bicarbonate buffered solution, pH 7.4, in equilibrium with a gas mixture of 95% O_2, 5% CO_2, containing K^+, Na^+, Mg^{++}, and Ca^{++} in concentrations of 70, 76, 6, and 2 meq./liter respectively. The medium for the adipose tissue was similar, but also contained succinate (0.01 *M*) and lacked calcium. Duplicate preparations from the same animal were set up simultaneously; one of these preparations was supplemented by the addition of glucose to the medium to a concentration of 400 mg.%. At the end of the incubation period the metabolic carbon dioxide produced was trapped in 30% KOH and from this were prepared infinitely thick plates of barium carbonate which, when counted, gave a measure of the conversion of the acetate carbon to carbon dioxide. The tissues were then hydrolyzed, the fatty acids separated, plated, and counted as plates corrected to a weight of 15 mg.

Nitrogen determinations were also made on both liver and adipose tissue from each animal by the micro-Kjeldahl method. The incorporation of acetate into fatty acids and carbon dioxide could then be expressed per mg. nitrogen in the specimens.

The duplicate tissue preparations, one with and one without glucose, from control and fasted animals were analyzed statistically as paired data.

Results

In Table I are shown the results of incubating liver slices from normal and fasted rats with 1-C^{14} acetate. It will be seen that fasting for 24 hours at 5° C. caused a marked impairment in acetate incorporation into fatty acids and carbon dioxide. Supplementation of the medium with glucose enhanced acetate incorporation into fatty acids by normal liver slices but did not affect the amount of carbon dioxide derived from acetate. In the cold fasted rats glucose supplementation greatly enhanced acetate incorporation into both fatty acids and carbon dioxide but in neither was it restored to normal levels.

Somewhat similar results were obtained on incubating liver slices with 2-C^{14} acetate, as shown in Table II. Acetate incorporation into liver fatty acids was only slightly enhanced with normal slices following the addition of

glucose to the medium; carbon dioxide production was unaffected. Slices from fasted animals incorporated much less of the methyl labelled carbon into fatty acids and carbon dioxide than did normal slices, but addition of glucose to the medium markedly enhanced these processes and in the case of carbon dioxide production restored it to normal levels.

The data also suggest that in both normal and fasted animals, although there was little difference in the percentage of acetate carbon which was incorporated into fatty acids from either the carboxyl or the methyl carbon, a much larger proportion of the carboxyl carbon than of the methyl carbon followed the oxidative pathway, especially in the slices from normal animals.

In Table III are set out data on the incorporation of the two differently labelled acetates into fatty acids and carbon dioxide by adipose tissue from these same animals. The results differ somewhat from those noted with

TABLE I

INCORPORATION OF 1-C^{14} ACETATE INTO FATTY ACIDS AND CARBON DIOXIDE BY LIVER SLICES. MEAN VALUES ± S.D. S.E.* =STANDARD ERROR OF MEAN DIFFERENCE BETWEEN PAIRS

	Normal (6)				Cold fasted (7)			
	Control	+ Glucose	S.E.*	*P*	Control	+ Glucose	S.E.*	*P*
Lipogenesis								
Cts./g. liver	38,600 ±6,200	44,900 ±5,000	2300	<.05	1,190 ±187	1,830 ±240	240	<.05
S.A. cts./15 mg. fatty acids	31,300 ±5,500	40,780 ±5,000	2500	<.02	617 ±125	961 ±210	140	=.05
Cts./mg. liver N	1,450 ±220	1,730 ±200	103	<.05	45 ±8	63 ±10	4	<.02
$C^{14}O_2$ production								
Cts./g. liver	70,700 ±16,000	60,150 ±7,600	6500	>.1	27,060 ±12,000	37,200 ±10,700	2340	<.01
Cts./mg. liver N	2,700 ±240	2,640 ±96	210	>.2	1,000 ±400	1,460 ±210	213	<.05

TABLE II

INCORPORATION OF 2-C^{14} ACETATE INTO FATTY ACIDS AND CARBON DIOXIDE BY LIVER SLICES. MEAN VALUES ± S.D. S.E.* =STANDARD ERROR OF MEAN DIFFERENCE BETWEEN PAIRS

	Normal (5)				Cold fasted (6)			
	Control	+ Glucose	S.E.*	*P*	Control	+ Glucose	S.E.*	*P*
Lipogenesis								
Cts./g. liver	36,000 ±9,800	44,900 ±11,700	4000	>.05	1,660 ±220	2,380 ±290	110	<.01
S.A. cts./15 mg. fatty acids	32,400 ±7,900	43,000 ±10,100	4080	=.05	1,350 ±270	1,960 ±330	145	<.02
Cts./mg. liver N	1,200 ±270	1,530 ±250	120	=.05	62 ±11	85 ±12	7	<.02
$C^{14}O_2$ production								
Cts./g. liver	27,180 ±4,800	24,620 ±3,000	1300	>.1	17,180 ±1,940	24,180 ±5,300	1750	<.02
Cts./mg. liver N	1,060 ±270	960 ±200	60	>.1	640 ±170	890 ±280	80	<.02

liver tissue. Fasting greatly inhibited the incorporation of both 1- and 2-C^{14} acetate into fatty acids, and to a lesser extent into carbon dioxide. Addition of glucose to the medium greatly stimulated fatty acid formation by both normal and fasted slices (in slices from the fasting animals the acetate incorporation into fat following glucose supplementation was similar to that found with normal slices without supplementation). The production of carbon dioxide from either 1-C or 2-C^{14} acetate by the adipose tissue was, in contrast to production by liver, unaffected by the addition of glucose.

TABLE III

INCORPORATION OF C^{14} ACETATE INTO FATTY ACIDS AND CARBON DIOXIDE BY ADIPOSE TISSUE. MEAN VALUES ± S.D. S.E.* = STANDARD ERROR OF MEAN DIFFERENCE BETWEEN PAIRS

1-C^{14} ACETATE

	Normal (6)				Cold fasted (7)			
	Control	+ Glucose	S.E.*	*P*	Control	+ Glucose	S.E.*	*P*
				Lipogenesis				
Cts./mg. N	2,390 ±360	22,160 ±7,600	190	<.001	127 ±70	2,300 ±1,200	1,030	<.05
S.A. cts./15 mg. fatty acids	580 ±210	4,900 ±1,600	170	<.001	97 ±90	810 +400	280	<.05
				CO_2 production				
Cts./mg. N	5,200 ±2,300	4,500 ±2,000	560	>.1	3,000 ±900	3,200 ±1,770	1050	>.2

2-C^{14} ACETATE

	Normal (5)				Cold fasted (6)			
	Control	+ Glucose	S.E.*	*P*	Control	+ Glucose	S.E.*	*P*
				Lipogenesis				
Cts./mg. N	536 ±90	12,660 ±5,000	4,700	<.05	30 ±7	1,480 ±500	540	<.05
S.A. cts./ 15 mg. fatty acids	490 ±85	10,000 ±4,020	3,600	<.05	32 ±5	1,370 ±460	510	<.05
				CO_2 production				
Cts./mg. N	3,660 ±760	2,800 ±860	905	>.2	2,920 ±1,940	2,990 ±3,400	90	>.2

It was noted with adipose tissue, as with liver, that a greater proportion of carboxyl carbon compared with methyl carbon followed the oxidative pathway in the normal animals. This difference was not apparent with adipose tissue from fasted animals. A point of additional interest noted in all these experiments was that, when acetate incorporation was compared on the basis of tissue nitrogen, adipose tissue was a much more active tissue than liver as regards acetate oxidation and lipogenesis.

Discussion

The above findings confirm the reports of Masoro *et al.* (4, 5) and extend the observations to include studies with acetate labelled on the methyl carbon and to studies with adipose tissue. Both liver and adipose tissue require carbohydrate for lipogenesis from acetate to proceed optimally. This action

of carbohydrate metabolites would not appear to be simply a matter of supplying a deficiency of precursors of acetate for, although fasting greatly reduces the carbohydrate stores, adding glucose to the medium undoubtedly enlarges the size of the acetate pool available for metabolism and would result in dilution in the proportion of the labelled compound in the pool, particularly in the fasted animals, and on this basis one would expect less incorporation of the label. Addition of carbohydrate has some other function than just enlarging the pool of available metabolite. As Masoro *et al.* have indicated (5), since pyruvate is also effective in restoring the defects of fasting, carbohydrate has its action at some place in glucose metabolism subsequent to the formation of pyruvate, possibly where acetate is converted to acetyl coenzyme A, in which form it undergoes its various reactions. However, Masoro *et al.* further demonstrated (5) that Krebs cycle intermediates were ineffective in stimulating acetate oxidation in the fasted animal, and hence the precise role and site of carbohydrate action are still obscure.

Fasting reduces acetate oxidation to carbon dioxide by adipose tissue as well as by liver but, unlike liver, addition of glucose does not affect carbon dioxide production by adipose tissue from fasted animals though it markedly stimulates fat synthesis. Possibly a greater concentration of glucose is required by adipose tissue but the work of Milstein and Hausberger (8) suggests that the glucose requirements of adipose tissue for purposes of lipogenesis and oxidation are considerably less than those of liver. This lack of carbohydrate, or energy derived therefrom, would appear to be of greater importance in liver tissue as there is no evidence that there is any marked impairment in the ability of adipose tissue from cold fasted animals to oxidize acetate or that acetate oxidation is stimulated by supplementation of the tissue with glucose. This might be associated with the inherently high metabolic activity of adipose tissue for, as shown above, compared per mg. of tissue nitrogen, it is a much more active tissue than liver.

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POLYGLYCERYLPHOSPHORIC ACIDS

I. SYNTHESIS OF BIS(L-α-GLYCERYL) PHOSPHORIC ACID[1]

ERICH BAER AND DMYTRO BUCHNEA

Abstract

Bis(L-α-glyceryl) phosphoric acid has been prepared by synthesis. It was obtained by phosphorylating D-acetone glycerol with phenylphosphoryl dichloride in the presence of pyridine, and freeing the intermediate product, bis(acetone L-α-glyceryl) phosphoric acid phenyl ester, of its protective groups by catalytic hydrogenolysis and mild acid hydrolysis.

In 1941, Pangborn (19, 20) reported the isolation from beef heart of a complex phosphatide that was free of nitrogen and appeared to be a mixed fatty acid ester of a polyglycerylphosphoric acid. Somewhat later, the presence of similar compounds was reported in the phosphatides of egg yolk by Fleury (15), and of liver by McKibbin and Taylor (17). Four years ago, Garcia, Lovern, and Olley (16,18), investigating the lipids of the flesh of cod and haddock, succeeded in isolating ethanol-soluble, nitrogen-free phosphatides of the bisphosphatidic acid type, in which fatty acids, glycerol, and phosphoric acid were present in ratios of approximately 4:2:1. More recently, Benson and Maruo (8, 9) reported the isolation from the alga *Scenedesmus* of ethanol-soluble phosphatides that were also free of nitrogen, but contained fatty acids, glycerol, and phosphoric acid in the ratios of 2:2:1, and which proved to be asymmetrically substituted fatty acid esters of bis(α-glyceryl) phosphoric acid. These phosphatidyl glycerols, which represent a new class of phosphatides, bear a strong structural resemblance to the phosphatidyl inositols isolated by Faure and Morelec-Coulon from wheat germ (11) and heart muscle (12).

Although it has not yet been possible to assign a definite configuration to the phosphatidyl moieties of phosphatidyl glycerols or phosphatidyl inositols, results of recent investigations concerning the configuration of naturally occurring glycerolphosphatides (4) suggest that these moieties possess the L-configuration. The same configuration may have to be assigned also to the fatty acid-free glycerol moiety of the phosphatidyl glycerols, if they prove to be formed, as proposed by Benson and Maruo (8), by an enzymatic transfer of glycerophosphoric acid (GPA) from cytidine diphosphate glycerol (CDPG) to a diglyceride, and if, as one would expect, the GPA-moiety of CDPG possesses the L-configuration. Thus it is possible that the naturally occurring phosphatidyl glycerols, as well as the closely related bisphosphatidic acids, may be found to be derivatives of bis(L-α-glyceryl) phosphoric acid.

The synthesis of several fully substituted fatty acid esters of bis(L-α-glyceryl) phosphoric acid was reported by one of the authors (E.B.) 5 years ago (2), and while it is probable that the bisphosphatidic acids isolated by

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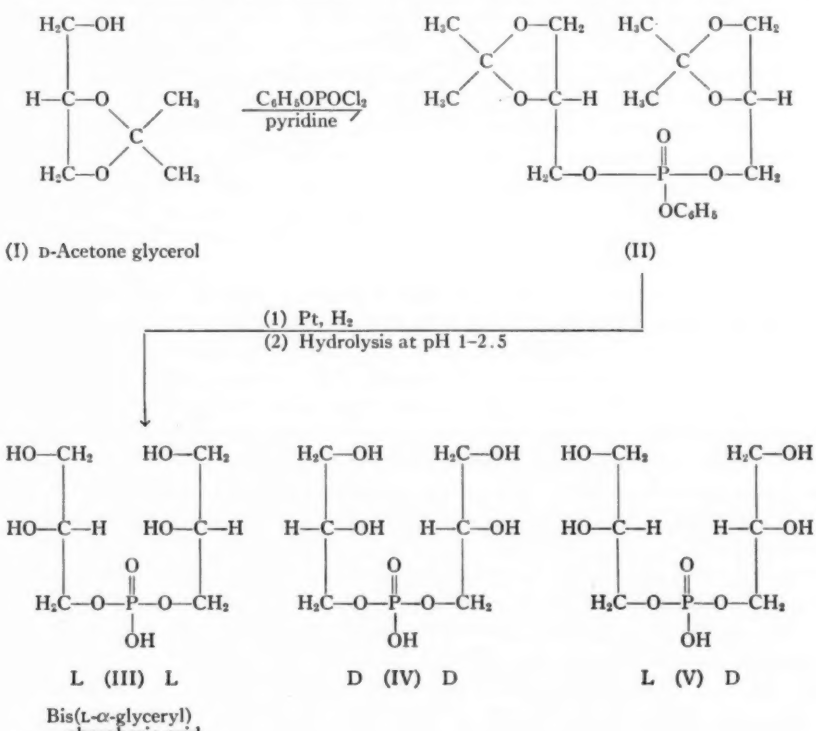
Garcia, Lovern, and Olley (16, 18) from the flesh of cod and haddock have the same configuration, this has yet to be proved. There are two general methods by means of which the configuration of a naturally occurring phosphatide can be determined. One requires the synthesis of the phosphatide with the expected structure and configuration for comparison with the natural product, while the other involves the degradation of the natural phosphatide to a product whose structure and configuration either is known or can be readily established. For the phosphatidyl glycerols and bisphosphatidic acid, the bisglycerylphosphoric acid would appear to be the most convenient stereochemical reference compound, since its enantiomers are readily synthesized by methods that clearly reveal their stereochemical relationship to D- and L-glyceraldehyde. Bis(α-glyceryl) phosphoric acid was prepared synthetically many years ago (7, 13) but was obtained only as an optically inactive mixture consisting presumably of the L,L (III), D,D (IV), and L,D (V)-isomers. In the experimental part of this paper we report the preparation of the L,L-isomer (III), which we believe is the one most likely to occur in nature. It was obtained essentially by the method of Fischer and Pfähler (13) for the



Reaction Scheme

synthesis of the racemic mixture of bis(α-glyceryl) phosphoric acids, except that we substituted D-acetone glycerol[2] and phenylphosphoryl dichloride for DL-acetone glycerol and phosphorus oxychloride (see Reaction Scheme), and removed the protective phenyl group of the intermediate product, bis-(acetone L-α-glyceryl) phenylphosphate (II) by catalytic hydrogenolysis. The bis(L-α-glyceryl) phosphoric acid, a viscous liquid, was obtained in an over-all yield of 77%. Its analytical values for carbon, hydrogen, and phosphorus agree well with those required by theory. On titration with periodate, it consumed 97.5% of the amount calculated for the presence of two pairs of vicinal hydroxyl groups per one molecule of the diester. The removal of the protective groups thus proceeds without migration of phosphoric acid. Its enantiomer, bis(D-α-glyceryl) phosphoric acid (IV), can be obtained by the same procedure with L-acetone glycerol (6) as starting material.

Experimental

Bis(acetone L-α-glyceryl) Phenylphosphate (II)

In a thoroughly dried three-necked 1 liter flask equipped with an oil-sealed mechanical stirrer, calcium chloride tube, and dropping funnel were placed 66.1 g. (0.5 mole) of D-acetone glycerol (3) and 100 ml. of glass beads (approximately 6 mm. in diameter). The flask was immersed in a water bath at 25°, and to the rapidly stirred mixture were added in a thin stream 80 ml. of anhydrous pyridine, followed by 52.8 g. (0.25 mole) of phenylphosphoryl dichloride (10, 22) over a period of 20 minutes. After 1 hour, the reaction mixture was diluted with 250 ml. of anhydrous ether, and the stirring was continued for another hour. The glass beads were then removed by filtration, washed with ether, and to the combined filtrates was added 500 ml. of ether. The precipitate, consisting of pyridine hydrochloride, was removed by filtration with suction, and the filtrate was washed rapidly with three 500 ml. portions of ice-cold 5 *N* sulphuric acid, two 500 ml. portions of a saturated sodium bicarbonate solution, and three 500 ml. portions of distilled water. The ethereal solution was dried with 200 g. of anhydrous sodium sulphate, the solvent was removed by distillation under reduced pressure, and the residue was kept in a high vacuum until constant weight was reached. The bis(acetone L-α-glyceryl) phenylphosphate, a viscous liquid, weighed 78.1 g. (77.6% of theory); n_D^{25} 1.4870 $[\alpha]_D^{25}$ +2.35° in substance, $[\alpha]_D^{25}$ +4.1° in benzene (*c*, 10). Anal. Calc. for $C_{18}H_{27}O_8P$ (402.4): C, 53.72; H, 6.76; P, 7.71; acetone, 28.87. Found:[3] C, 53.44; H, 6.74; P, 7.68; acetone, 28.20.

[2]The phosphorylation of D-acetone glycerol introduces the phosphoric acid at the hydroxy-group that has been formed by the reduction of the carbonyl group of acetone D-glyceraldehyde. Hence if one were to oxidize the terminal hydroxyl groups of bis(α-glyceryl) phosl phoric acid (III), bis(L-glyceraldehyde)-3-phosphate would be formed. The bis(α-glyceryl) phosphoric acid III thus by definition (14) belongs to the L-series.

[3]The carbon and hydrogen values were obtained by combusting the phosphate esters in the presence of vanadium pentoxide.

Bis(L-*α*-*glyceryl*) *Phosphoric Acid* (*III*)

Removal of the Phenyl Group by Catalytic Hydrogenolysis

A solution of 28.2 g. (0.07 mole) of bis(acetone L-α-glyceryl) phenylphosphate in 500 ml. of 99% ethanol, and 3.5 g. of platinum oxide (Adams catalyst)[4] were placed in an all-glass hydrogenation vessel of 1.5 liter capacity, and the mixture was shaken vigorously in an atmosphere of pure hydrogen[5] at a positive pressure of approximately 50 cm. of water until the absorption of hydrogen ceased. The hydrogenolysis was complete at the end of 1 hour. After the hydrogen had been replaced with nitrogen, the mixture was filtered, the catalyst was washed with a small amount of ethanol, and the combined filtrates were concentrated *in vacuo* to a volume of approximately 100 ml.

Deacetonation

The concentrate was diluted with 50 ml. of distilled water; to the strongly acidic solution (pH 0.85) was added 30 g. of Amberlite IR-120 (H), and the mixture was shaken for 1 hour at room temperature. The Amberlite was removed by filtration, the filtrate was cleared by centrifugation, and the solvents were removed by distillation *in vacuo* at a bath temperature of 35°–40°. The residue was freed of the last traces of solvents at a pressure of 0.1 mm. Hg. The remaining bis(L-α-glyceryl) phosphoric acid (III), a water-clear, viscous liquid, weighed 16.5 g. (95.8% of theory). The over-all yield was 77.4% calculated from D-acetone glycerol. The bis(L-α-glyceryl) phosphoric acid was found to be readily soluble in water, ethanol, methanol, but insoluble in ether, acetone, chloroform, or dimethylformamide; n_D^{22} 1.4825, n_D^{25} 1.4788; pH of a 0.2 molar solution of the diester in water, 1.15. Anal. Calc. for $C_6H_{15}O_8P$ (246.2): C, 29.27; H, 6.14; P, 12.58. Found:[3] C, 29.50; H, 6.20; P, 12.52.

Optical Activity

The bis(L-α-glyceryl) phosphoric acid in a freshly prepared solution in water (*c*, 10) or 99% ethanol (*c*, 10) possessed the specific rotations of $[\alpha]_D$ $-1.7°$ and $[\alpha]_D$ $-7.6°$, respectively. The rotation of an ethanolic solution of the diester (*c*, 10) kept at room temperature changed gradually from α_D $-0.76°$ (1 dcm. tube) to $-0.56°$, $-0.53°$, $-0.50°$, $-0.45°$, $-0.35°$, $-0.27°$, and $-0.26°$ at the end of the 1st, 2nd, 5th, 10th, 14th, 18th, and 24th days, respectively.

Vicinal–Glycol Titration with Periodic Acid

The titration (5) of bis(L-α-glyceryl) phosphoric acid was carried out on 20-ml. aliquots of a solution containing 553.0 mg. (2.246 millimoles) of the acid in 100 ml. of water that had been adjusted to pH 4.2 by the addition of sodium bicarbonate. After 18 hours, 0.449 millimole of the diester had consumed 0.875 millimole of periodic acid, i.e. 97.4% of the theoretical amount calculated for bis(L-α-glyceryl) phosphoric acid.

[4]The catalyst was prepared as described by Adams, Voorhees, and Shriner (1), with the exception that the sodium nitrate was replaced by an equimolecular amount of potassium nitrate.

[5]Electrolytically produced hydrogen was used.

Barium Bis(L-α-*glyceryl*) *Phosphate*

A 28.2 g. (0.07 mole) quantity of bis(acetone L-α-glyceryl) phenylphosphate was freed of the protective phenyl group by catalytic hydrogenolysis as described above. After the catalyst had been removed, the ethanol was distilled off *in vacuo* at a bath temperature of 35°–40°, the residue was dissolved in 25 ml. of distilled water, and to the solution was added gradually a saturated aqueous solution of barium hydroxide until its pH was 2.5. After the solution had been standing for 12 hours at room temperature, its pH was adjusted to a value between 8 and 9 by addition of barium hydroxide. The excess of barium ions was precipitated with carbon dioxide, the barium carbonate was removed by centrifugation, and the solution was concentrated *in vacuo* to one half of its volume at a bath temperature of 40°–45°. The concentrate was cleared by centrifugation, and the barium salt was precipitated by the gradual addition of ethanol. The precipitate was centrifuged down, washed with anhydrous ethanol, and dried *in vacuo* over phosphorus pentoxide until its weight was constant. The sponge-like material, which could be pulverized, was very hygroscopic and in the presence of moisture formed a glass-like material. The barium bis(L-α-glyceryl) phosphate was obtained in a yield of 19.5 g. (88.7% of theory). It was found to be readily soluble in water, or dimethyl formamide, but insoluble in the more common organic solvents; $[\alpha]_D$ $-0.5°$ in 1 *N* hydrochloric acid (*c*, 10). Anal. Calc. for $[C_6H_{14}O_8P]_2Ba$ (627.7): C, 22.96; H, 4.50; P, 9.87; Ba, 21.88. Found:[3] C, 22.63; H, 4.52; P, 9.91; Ba, 21.87.

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Some years ago, preliminary work on the synthesis of bis(L-α-glyceryl) phosphoric acid was carried out in this Department by Dr. Alan G. Newcombe. His contribution is gratefully acknowledged.

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